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Internship at the sole hatchery Safiestela, S.A. Substitution of Instar I by enriched Instar II *Artemia* in the first days of *Solea senegalensis* rearing

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List of Abbreviatures

ARA – Arachidonic acid

DAH – Days after hatching

DHA – Docosahexaenoic acid

DPA – Docosapentaenoic acid

D_W – Dry weight

EPA – Eicosapentaenoic acid

FA – Fatty acid

FAA – Free amino acids

FAME – Fatty acid methyl esters

HUFA – Highly unsaturated fatty acid

IEM – Eye migration index

K – Condition index

KOH – Potassium hydroxide

L_{ST} – Standard length

OA – Oleic acid

PUFA – Polyunsaturated Fatty acid

RAS – Recirculating aquaculture system

RGR – Relative growth rate

SD – Standard deviation

SFA – Saturated Fatty acid

SGR – Specific growth rate

TFA – Total fatty acid

Abstract

Aquaculture is one of the fastest growing food sectors with a huge economic importance in several countries. European aquaculture has increased substantially in the last years with 35 aquatic species being produced. Production is dominated by rainbow trout, Atlantic salmon, sea bream and sea bass.

Portugal attended with 5760 tons in 2014 and most cultured species were turbot, sea bream, rainbow trout, sea bass and sole. However some of these species already show signs of market saturation.

Solea senegalensis presents itself as a good alternative as a native species with high market value and a biological cycle that can be completed in captivity. Its culture has been possible for several years, but intensive production has been slow to take off due to high mortalities at weaning, diseases, variable growth and poor juvenile quality. However in the last years improved practices and management have boosted the cultivation of *S. sole* to become a knowledge-driven sustainable industry.

Safiestela - Sustainable Aqua Farming Investments, S.A. is a company responsible for the mass production of juveniles of *Solea senegalensis*. It's the only *Solea senegalensis* hatchery in Portugal and belongs to the Spanish group Sea8. Its objective is to satisfy the market demand of this species generated by diminishing fisheries landings of sole.

The objective of this thesis was to perform a professional internship in this company "Safiestela, S.A." and have the opportunity to work in the daily routines of different stations and sections of sole production cycle. These include broodstock management, eggs, larval rearing, live food production, sole rearing and overall systems maintenance. The internship was carried out for 7 months in the facilities of the company located in Portugal (Lat. 41,452457; Long. -8,7721) from October 2014 to April 2015, followed by a trial to test the effects of the substitution of *Artemia* strains in the larval feeding protocols of the company.

I tested the effects of substituting the use of non-enriched *Artemia sp.* AF nauplii during first days of feeding in *Solea senegalensis* by an early co-feeding with enriched *Artemia sp.* EG metanauplii. Different dietary treatments significantly affect growth in sole larvae, with the treatment group (early co-feeding with enriched *Artemia sp.* EG)

displaying higher dry weight and better size homogeneity in tanks populations by the end of the trial. This group also display less incidence of deformities compared to the control.

It was concluded that the early co-feeding between rotifers and enriched *Artemia* metanauplii is possible, with positive results for larval development as it enhanced larval weight, homogeneity in the tanks and reduced the incidence of deformities.

Keywords: *Artemia sp.*, *Brachionus plicatilis*, *Solea senegalensis*, larvae

Resumo

A aquacultura é um dos sectores alimentares com maior crescimento, com grande importância económica em vários países. A produção europeia tem aumentado nos últimos anos com 35 espécies cultivadas, nomeadamente truta arco-íris, salmão, robalo e dourada.

Portugal produziu 5760 toneladas em 2014 e as espécies mais cultivadas foram pregado, truta arco-íris, robalo, dourada e linguado. No entanto algumas destas espécies já começam a mostrar sinais de saturação no mercado.

Solea senegalensis apresenta-se como uma boa alternativa, dado ser uma espécie nativa com elevado valor comercial e cujo ciclo biológico pode ser facilmente reproduzido em cativeiro. O seu cultivo é possível há vários anos, contudo a evolução da produção intensiva desta espécie tem sido lento, devido a mortalidades elevadas durante o desmame, doenças, crescimento variável e fraca qualidade de juvenis. No entanto, nos últimos anos, melhorias nas práticas e gestão do cultivo desta espécie impulsionaram a produção do linguado.

Safiestela - Sustainable Aqua Farming Investments, S.A. é uma empresa responsável pela produção em massa de juvenis de *Solea senegalensis*. É a única maternidade de linguado em Portugal e pertence ao grupo espanhol Sea8. O seu objectivo é satisfazer a procura no mercado desta espécie, gerada pela redução das pescas do linguado.

O objectivo desta tese foi realizar um estágio profissional na empresa “Safiestela, S.A.” e ter a oportunidade de trabalhar nas rotinas diárias das diferentes estações e secções da produção do linguado. Incluindo manutenção de reprodutores, ovos, cultivo larvar, produção de alimento vivo, engorda e manutenção dos sistemas de produção. O estágio durou 7 meses e foi feito nas instalações da empresa, localizadas em Portugal (Lat. 41,452457 Long. -8,7721) desde Outubro 2014 até Abril 2015, seguido de um ensaio para testar os efeitos da substituição de diferentes tipos de artémia nos protocolos larvares da empresa.

Testámos os efeitos da substituição de naúplios de *Artemia sp.* AF não enriquecidos nos primeiros dias de alimentação de *Solea senegalensis*, por um período de co-alimentação antecipado de rotíferos e metanaúplios enriquecidos de *Artemia sp.* EG. Os diferentes regimes alimentares mostraram diferenças significativas no

crescimento das larvas de *Solea senegalensis*, com o grupo tratamento (co-alimentação antecipada de *Artemia sp.* EG) exibindo um peso seco das larvas superior e melhor homogeneidade nos tanques no fim do ensaio. Este grupo também teve uma menor incidência de deformações no esqueleto, comparado com o grupo controlo.

Conclui-se que a introdução antecipada de metanaúplios enriquecidos de *Artemia sp.* EG no regime alimentar das larvas de *Solea senegalensis* é possível, com resultados favoráveis no desenvolvimento larvar, melhorando o peso das larvas, a homogeneidade nos tanques e reduzindo a incidência de deformações.

Palavras-chave: *Artemia sp.*, *Brachionus plicatilis*, *Solea senegalensis*, larvas

I. Introduction

1 Aquaculture review

In the last decades aquaculture production has increased considerably due to the demand of the world food supply, demographic expansion and overexploitation and exhaustion of fisheries resources.

In 2012 aquaculture accounted with 40% of food fish supply. It´s one of the fastest growing food sector with an annual growth rate of 6.2% between 2000 and 2012 (FAO, 2015).

Food fish supply is increasing at an average annual rate of 3.2%, surpassing, world population growth at 1.6%. This has boosted the world fish consumption as fish per capita in the 1960s was 9.9 kg and nowadays it reached 19.2 kg (FAO, 2015).

World population is expected to reach 9.6 billion people by 2050. This poses a challenge to produce enough food to feed the Planet while safeguarding natural resources for future generations (FAO, 2015)

World fisheries captures and aquaculture production supplied 158 million tons of food fish in 2012. Fisheries accounted with 91.3 million tonnes and aquaculture with 66.6 million tons, as shown in figure 1. Around 86% of world production was used for direct human consumption, with the remaining destined to non-food uses.

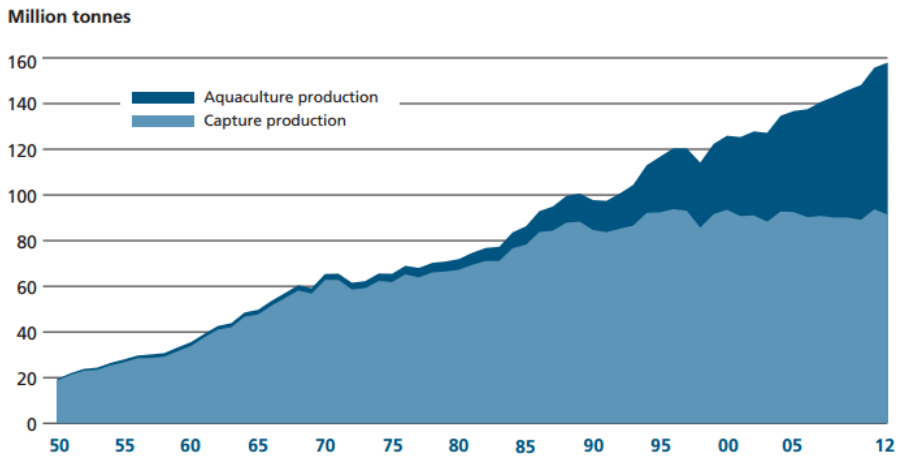

Figure 1 - World capture fisheries and aquaculture production (FAO, 2015)

World production is divided into three types of environment (freshwater, brackish water and marine), as it shows in figure 2. Marine production has one of the highest annual growth rates with 9.3% increase since 1990 to 2010 (Fisheries - European Commission, 2014).

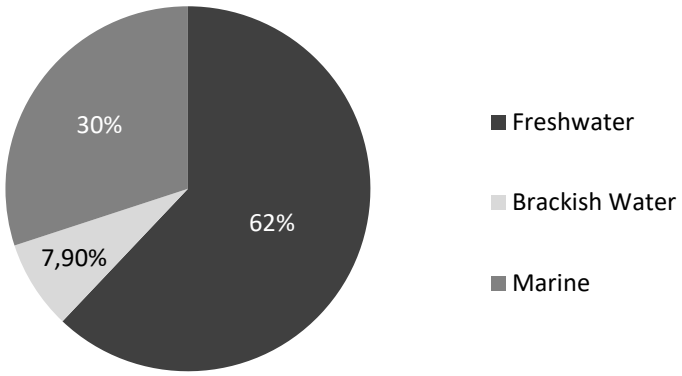

Figure 2 – Aquaculture world production by environment (Fisheries - European Commission, 2014)

EU is the 8th largest aquaculture producer in the world, with 1.25 million tons. Half of the production is based on molluscs and crustacean species, followed by marine fish (27%) and freshwater fish (23%). The most produced species in EU are mussel (*Mytilus edulis*), trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*). With a turnover of 3.5 billion euros, this sector employs around 85,000 people (Fisheries - European Commission, 2014).

Portugal attended with 5,760 tons in 2014 (FEAP, 2016). Most cultured species are clam (*Ruditapes decussatus*), oyster (*Crassostrea sp*.), mussel (*Mytilus edulis*), turbot (*Psetta maxima*), sea bream (*Spaurus aurata*), rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*).

Although some measures have been taken to reduce countries national fleets, with the increasing demographic growth and consequent overexploitation of marine resources, aquaculture stands as the only viable option for the supply of fish protein.

Environmental consequences of the aquaculture fast growth in the past are a major international concern. In order to reduce pressure on wild stocks and increase fish food supply, improved practices are crucial to a sustainable aquaculture.

2 Live feeds in Aquaculture

Zooplankton is a major part of the natural diet of several fish species (Evjemo *et al.*, 2003). Many species reared their entire life cycle in aquaculture aren't able to grow if exclusively fed on artificial diets during the first stages of life. Although recent studies show positive results with fish raised only on formulating diets, live feeds are still the best option for feeding the initial life stages of finfish species until weaning is possible (Fernandez-Diaz and Yúfera, 1997; Cahu and Infante, 2001).

In first feeding, fish larvae mostly depend on ocular stimulation to find food. Live moving preys are able to swim in the water column making them more attractive than inert food particles. Formulated diets tend to agglomerate on the water surface or the bottom of the tanks making them unavailable to larvae (Bengtson, 2003). Another advantage of live feeds is that the thin exoskeleton and high water content of live prey may be more palatable to the larvae, when compared to the dry formulated feds. Palate is important, as young fry capture food items and quickly accept or reject them based on palatability.

Proteins and free amino acids (FAA) are important for growth in all fish (Hilton *et al.*, 2008). FAA come as an absolute necessity to support growth and survival at first feeding, due to the fact that fish larvae have immature digestive systems and consequently can't break down protein (Govoni *et al.*, 1986; Helland *et al.*, 2003). Live preys have FAA and a large fraction of soluble, intact proteins. These are more convenient for larvae digestion and absorption than insoluble proteins (Srivastava *et al.*, 2006).

One of the biggest controversies in the live feed field is whether the use of cultured rotifers and *Artemia* are sufficient to produce good quality fry or if the supplementation with natural zooplankton or cultured copepods is necessary. Most cultured species have excellent results when fed with natural zooplankton, and some species, with small mouths, struggle to eat rotifers at first feeding due to their size. Thus copepods are a good alternative prey to these species, thanks to their high quality nutritional profile and small size. Yet, the mass production of copepods is difficult, and commercial-scale production has not yet been achieved (Støttrup, 2000).

Most common live prey used in rearing of marine fish larvae are rotifers (*Brachionus spp*.) and brine shrimp (*Artemia sp*.) (Evjemo and Olsen, 1997). These

species are easy to mass produce, making them convenient for commercial hatchery operations of all live preys available (Lie *et al.*, 1997; Conceição *et al.*, 2010).

3 Rotifers

Rotifers belong to a small group of unsegmented, pseudocoelomate aquatic invertebrates with bilateral symmetry.

Most forms are free-swimming (Ruttner-Kolisko, 1974; Pontin, 1978; Wallace *et al.*, 1991; Nogrady *et al.*, 1993), and they can constitute up to 30% of freshwater plankton biomass, making an important link between primary producers and predators, by consuming bacteria and algae.

Rotifers are identified by their anterior apical ciliated corona, which is responsible for swimming and feeding activities. Their body shape ranges from saccate to cylindrical and is divided into 4 sections.

Most rotifers eat by filter feeding, also called microphagus feeding (Pourriot, 1977; Clement *et al.*, 1983). They use the rotational movement of the corona, to direct the water flow into the mouth in order to capture food particles. Rotifers are usually mechanical non-selective grazers, however food selectivity can occur, but mainly by prey size preference (Rothhaupt, 1990a; Rothhaupt, 1990b), as their sensory receptors in the buccal tube can detect the size of particles (Clement *et al.*, 1983; Hansen *et al.*, 1997). Prey size can range between bacteria to dinoflagellates (Hansen *et al.*, 1997) and depends on rotifer body size (Hino and Hirano, 1980).

3.1 Reproduction

Females have a single gonad and are mostly oviparous, with embryos developing outside the maternal body.

Rotifers from the group *Brachionus* reproduce by cyclic parthenogenesis. Meaning that asexual reproduction is most common, but sexual reproduction can also occur. As a result most of rotifers in the wild are females, with males only appearing during short

periods of time. The type of reproduction is related with habitat conditions, with population rapidly increasing through diploid parthenogenesis during favourable conditions.

Females are diploid and have a bigger size when compared to males, which are haploid. There are two types of naturally occurring females (Figure 3), amitic and mitic. Amitic females produce parthenogenetically diploid eggs that develop by mitosis into females. Mitic females produce parthenogenetically haploid eggs by meiosis. If fertilized, these eggs develop into diploid resting eggs or cysts, which form diploid amitic females, while if not-fertilized, these haploid eggs develop into males (Dhert and Sorgeloos, 1995).

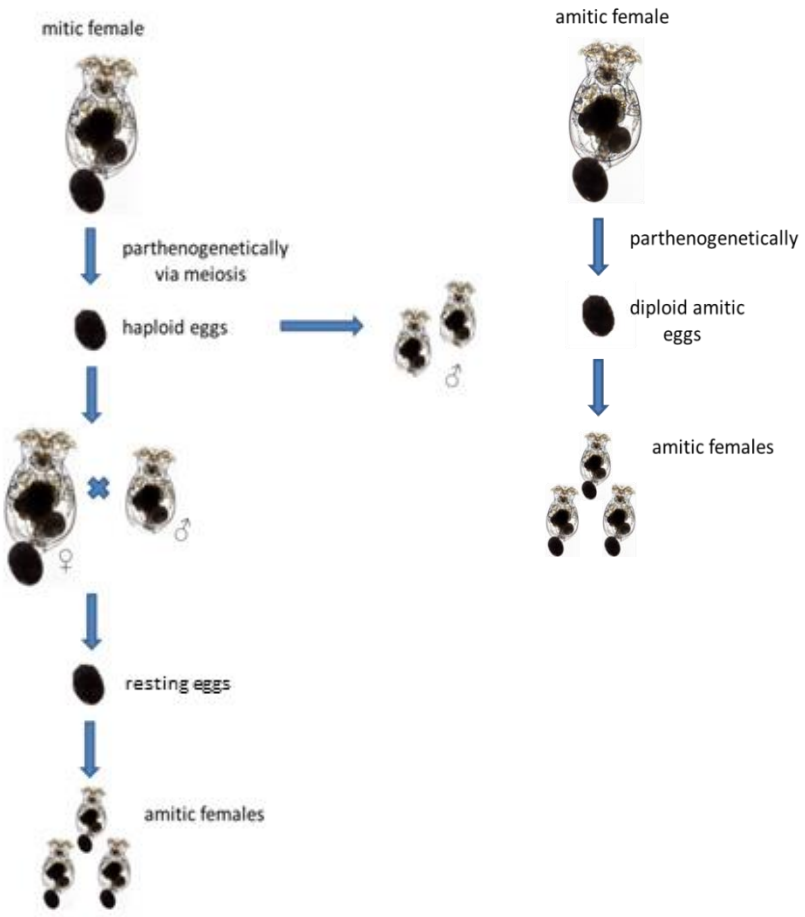

Figure 3 – Types of reproduction between mitic and amitic females in rotifers

Usually amitic or non-fertilized mitic eggs are carried outside of the female body. Resting eggs or cysts, depending on strain, can be carried outside or inside the maternal body (Serra *et al.*, 1998). Amitic eggs and male eggs hatch immediately, however resting

eggs take longer periods of time, and only hatch under certain conditions (Hagiwara, 1996; Lubzens *et al.*, 2001).

The fecundity of the population is related to the type of reproduction, but generally the production of amitic eggs is ten times faster than the production of resting eggs. Unfertilized mitic females don't contribute to the population growth as they produce males. (Dhert and Sorgeloos, 1995)

This type of reproduction allows rotifers to take advantage of both rapid population growth for colonization without genetic recombination and the genetic recombination ability, to form resting eggs. These have the opportunity to disperse into new areas and withstand, in a dormant state, for longer periods of time waiting for better environmental conditions to hatch. Resting eggs ensure that the population can survive periods of bad habitat conditions and ultimately maximize the population fitness (Serra and King, 1999).

The population mitic/amitic ratios waver in response to environmental conditions (density, food availability, salinity and temperature), and optimal conditions result in a higher production of resting eggs (Lubzens *et al.*, 1985; Snell, 1986; Snell and Boyer, 1988; Lubzens *et al.*, 1993; Serra and King, 1999).

3.2 Culture

Setting up a rotifer mass culture, starts with choosing the correct species and strain. This will determine the size of the prey, type of reproduction, reproductive rates and ultimately culture conditions.

Due to their fast reproductive cycle, rotifers can be cultivated in high production cultures in short periods of time, achieving extremely high densities, making them a convenient prey for commercial hatcheries. Culture reproductive rate is dependent on the type of reproduction taking place (Dhert and Sorgeloos, 1995). These ratios can be conditioned by environmental conditions like temperature, salinity and type or quality of food. For feeding purposes, rotifer cultures are encouraged to reproduce asexually, as the egg production rate in females can be up to 10 times faster than the production of resting eggs. Sexual reproduction also results in males, which are not desired, due to nutritious inferior quality and faster swimming patterns when compared to females. The inferior nutritional quality is due to males lacking a digestive system, making them incapable of being enriched with oil emulsions for larvae feeding. Sexual reproduction can be avoided

by manipulating culture conditions to encourage asexual breeding (high salinities) or by selecting specific genetic strains that do not breed sexually (Hino and Hirano, 1976; Hagiwara *et al.*, 1995).

The culture reproductive rate (r) increases exponentially with increasing food concentrations, with a threshold for maximum growth (Rothhaupt, 1990c). Beyond this point, increasing food concentration will not positively affect population growth, and may even decrease it. The type of food, quality and quantity will affect culture growth, with algae encouraging higher reproductive rates than yeast (Lubzens *et al.*, 2001).

The type of food will directly affect the operation costs and the amount of food will be dependent on culture density, temperature, rotifer species, salinities and reproduction rate (Dhert and Sorgeloos, 1995). This is determined by daily sampling the culture to determine the population size and the percentage of individuals carrying eggs.

3.3 Water quality

There are different culture methods, each one with a different density and production rates, with optimum culture conditions (varying between species and strains).

The optimal temperature for culture is species dependant, with optimal values for *B. picatilis* 10 - 30ºC and *B. rotundiformis* 24 - 35ºC (Hirano, 1987; Lubzens *et al.*, 1987; Lubzens *et al.*, 1989; Rumengan and Hirayama, 1990; Hirayama and Rumengann, 1993).

High density cultures must be aerated with pure oxygen in order to prevent low oxygen levels in the water, minimum 4 ppm (Fulks and Main, 1991).

The pH is the most important factor in the culture, as it is strictly related to the concentration and toxicity of nitrogen waste in the culture medium. The optimal range is between 7.5 - 8.5 (Hirano, 1987; Fulks and Main, 1991).

Ammonia levels must remain under 1 mgL^{-1} and nitrate under 6 - 10 mgL^{-1} (Fulks and Main, 1991). Higher pH will increase the amount of non-ionized ammonia (NH_3), which is a more toxic form of this nitrogen compound to rotifers and fishes. While lower pH will decrease the amount of toxic ammonia (NH_3), and increase the amount of its ionized form, NH_4^+, a less toxic form of ammonia. Fluctuations of the pH will occur during the day, as the strong aeration and food surplus will decrease the pH, increasing the levels of ammonia in the water. Lower pH will affect the nitrogen cycle, mainly the processing of

ammonia into nitrates by *Nitrossomas bacterium*. The conversion rates will decrease bellow 6 and if pH continues to decrease, the activity of these bacteria can be affected. This can lead to the accumulation of ammonia in the tank, encouraging bacterial growth. Therefore a strict balance between rotifer density and food supply must be kept to avoid food surplus in tanks, leading to accumulations of organic matter in the water.

Deterioration of water quality may induce bacterial growth with opportunistic pathogens that are common in seawater (Skjermo and Vadstein, 1999). Cultures with low oxygen concentrations may serve as medium for bacteria growth as *Vibrio spp*., that are infectious to fish and may dominate rotifer cultures bacterial assemblage, as they reproduce more quickly than other non-pathogenic bacteria. Pathogens are a main problem in these cultures, as bacteria free cultures are extremely difficult to achieve, due to ineffectiveness of sterilisation methods and antibiotics as a result of their small size (Maeda *et al.*, 1997; Rombaut *et al.*, 1999).

3.4 Rotifers as prey

A huge breakthrough in aquaculture was made in the 1960s with the discovery that rotifers were a convenient live food for both fresh and marine species. They could be culture as prey for species that could not first feed on *Artemia* due to their size, namely marine larvae (Hirata, 1979).

Rotifers have been used as food organism in marine production for four decades and although they are not the natural food of marine larvae, rotifer cultures can create a continuous, stable source of nutritional food, essential for a successful hatchery operation.

They are used in the first days of rearing, and mainly two species are cultured, *Brachionus rotundiformis* (known as S-type) and *Brachionus plicatilis* (L-type).

In commercial hatcheries, high density cultures provide up to billions of rotifers per day (Lubzens *et al.*, 2001), since each larvae can eat around 20,000 to 100,000 rotifers during first 20 - 30 days of culture (Ikenoue and Kafuku, 1992; Lubzens *et al.*, 2001).

4 *Artemia*

Artemia is found around the world in high salinity environments with specific ecological conditions that led to different geographical strains, within the same species. These environments with simple trophic structures and low species diversity combined with the absence of predators and food competitors, allowed the formation of monocultures of *Artemia*.

Artemia eggs hatch into the first larval stage, Instar I, with 400 - 500 µm in length, a brownish-orange colour, one red nauplius eye in the head and three pairs of appendices. Instar I does not feed and depends on its yolk reserves, due to a non-functional digestive system (Dhert and Sorgeloos, 1995).

After 8 hours Instar I molts into Instar II stage and starts to feed on small particles (1 - 50 µm). The larvae will continue to grow and molt into new stages, usually 1 nauplius, 4 metanauplius, 7 post-metanauplius and 5 post-larvae stages. On the 10th instar forward morphological and functional changes occur, as the antennae lose locomotion function and undergo sexual differentiation (Dhert and Sorgeloos, 1995).

Adults are primitive arthropods with 8 - 12 mm in length, with an elongated linear and segmented body. They possess two eyes, a linear digestive system, sensorial antennae, a pair of functional theracopods in each of the eleven thoracal segments and a furca on the last body segment (Figure 4). Their body is covered with a thin and flexible exoskeleton (Dhert and Sorgeloos, 1995).

In order to thrive in harsh environment *Artemia* have an efficient osmoregulatory system to withstand high salinities, capacity to synthetize efficient respiratory pigments to survive in low oxygen conditions and produce dormant eggs (cysts) (Dhert and Sorgeloos, 1995) during suboptimal environmental conditions to ensure the survival of the species.

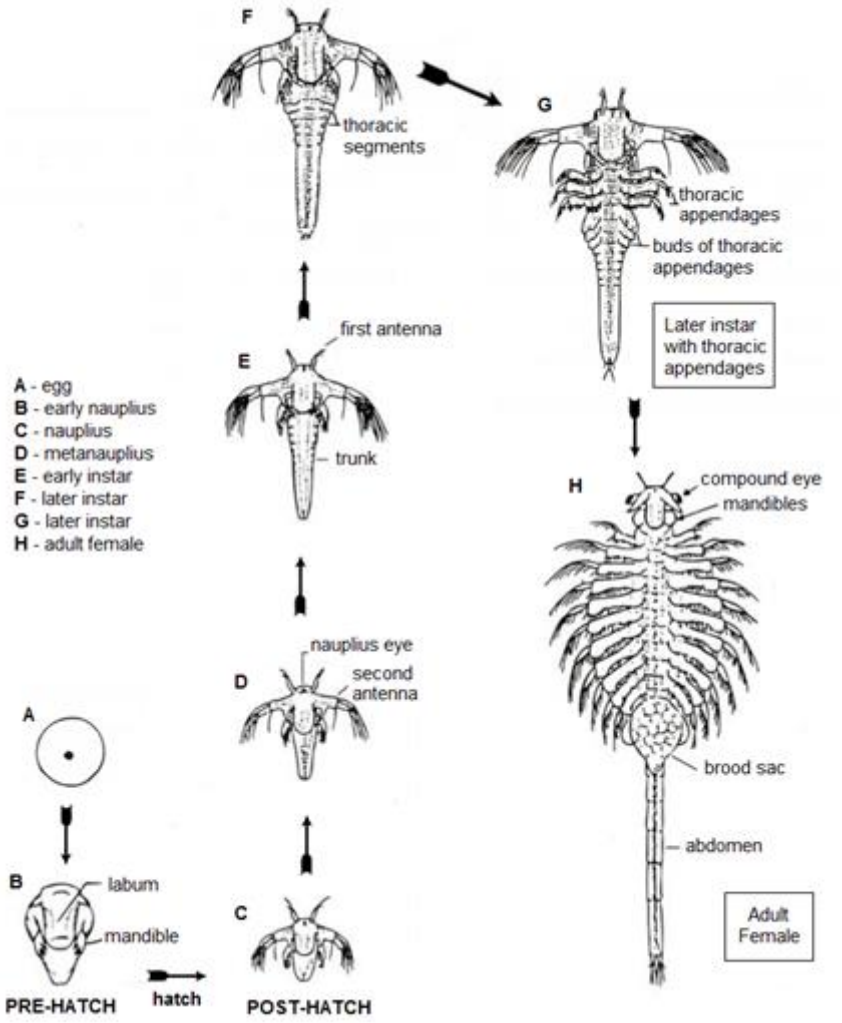

Figure 4 - *Artemia* development from cyst to adult. Adapted from Drewes (2005).

4.1 Cysts

Artemia females can alternate between production of live nauplii (ovoviparity) and production of dormant eggs (oviparity).

Cysts are in a dormant stage, diapause, designed to overcome temporary harsh environmental conditions, that also allows synchronization of population development in analogously to habitat changes, as they can survive through extreme conditions (high salinities, high temperatures and desiccation).

Dormant eggs float in the water column and spread through space and time, remaining in an inactive metabolic state, while kept dry. They are bioconcave, and when immerse in water start to hydrate and become spherical within 2 hours. When water levels

reach 30 - 65%, diapause is deactivated and embryo metabolism is resumed, with respiration, RNA and protein synthesis starting within minutes. After an 8 - 24 hours period, depending on salinity and temperature, the embryo emerges shrouded with a hatching membrane, and it's called the umbrella stage (Dhert and Sorgeloos, 1995). Shortly after the membrane breaks and a free swimming nauplii comes out.

4.2 Culture

Hatching success depends mainly on techniques and conditions used for harvesting, hygiene, drying cysts and storage.

During diapause, embryo metabolism is suspended as long as cysts remain dry, so storage conditions should avoid high humidity, as the embryo will reinitiate its metabolism around 30 - 65% water content, compromising cyst quality. Humidity fluctuations will lead to depletion of yolk reserves and ultimately decrease hatchability, due to the lack of energy to complete hatching (Dhert and Sorgeloos, 1995). Hydration above 65% will lead the embryos to complete their pre-emergence state and a new dehydration will result in the death of the now differentiated embryo (Dhert and Sorgeloos, 1995).

Culture conditions can also affect *Artemia* hatchability:

A constant temperature of 25 - 28ºC should be maintained (Dhert and Sorgeloos, 1995);

The pH should be kept in the 8 - 8.5 range, for maximum hatching enzymes activity (Dhert and Sorgeloos, 1995);

Hydration of the cysts is salinity dependent, as high salinities will take long to hydrate the cyst, and low salinities result in faster hydration. A range between 15 - 35 ppt is commonly used to provide better hatching results;

Incubation should be performed with 2 gL^{-1} of cysts and to support these high densities oxygen levels should be kept around 5 mgL^{-1}. Too much oxygen can result in pale animals, due to the low production of haemoglobin (Dhert and Sorgeloos, 1995);

A minimum of 2000 lux is required to trigger hatching in hydrated cysts (Dhert and Sorgeloos, 1995).

After hatching, *Artemia* should be separated from their hatching wastes (i.e. empty or unhatched cysts). This step is time consuming and labour intensive and if not properly done can lead to issues in production, as empty shells can lead to mortality in small larvae. However a new generation of commercial products based on new magnetic coating technology of cysts, has reduced the time and labour of this step in *Artemia* production. Magnetic coated cysts are fully separated from free swimming nauplii using a separator tube with magnets, resulting in a clear separation between nauplii and empty or unhatched cysts in a short period of time. These products provide a better optimization of cysts usage and yield more nauplii biomass, as they can gentle separate a full tank with no use of chemicals (decapsulation) or induce physical stress to the nauplii.

The separated *Artemia* should be transferred to a new tank and be enriched for 24 hours prior to feeding them to the larvae.

Nutritional quality can be manipulated either by enrichment techniques or by the selection of different strains. *Artemia* is deficient in fatty acid profile, so enrichment is required when using post-instar I stages. Enrichment is possible due to the continuous non-selective filter feeding behaviour of brine shrimp. As a result, instar II usually reflects the lipid profile of their diet (Dhert and Sorgeloos, 1995).

4.3 Cold Storage

Young larvae require several meals per day, with specific size preys. As *Artemia* size and nutritional quality varies among life stages, this poses a challenge to deliver a constant supply of same size and constant quality of *Artemia* throughout the day. In order to achieve this, several hatchings/harvestings a day would be necessary in a commercial hatchery. The cold storage of artémia in a refrigerated container is possible with no negative results in mortality and reduces the hatching efforts in the hatchery, with only one hatch and one harvest per day. It also helps reducing retention times in larvae tanks, granting a better water quality, less food surplus and avoids loss of nutritional quality of the prey. Also cold storage *Artemia* is less active, making it easy to capture by the larvae due to its slow swimming speed.

Instar I stage does not feed and depends on yolk reserves until it molts to the next stage. As a result the quality of Instar I declines overtime due to metabolism, and eventually molts into Instar II. When using non enriched *Artemia*, Instar I quality is richer

than starving Instar II, so cold storage can be used for Instar I to delay metabolism losses and moulting into Instar II.

When using enriched *Artemia* Instar II forward, after harvesting *Artemia* content can change due to its own metabolism, though not reflecting its diet profile. As up to 70% of DHA gain in enrichment can be catabolized into EPA by the *Artemia* (Estevez *et al.*, 1998), with conversion rates varying between enrichments and temperatures (Bell *et al.*, 2003). Cold storage avoids content losses of enriched *Artemia* by reducing its metabolism, thus enhancing enrichment process.

Artemia can be kept at 5ºC at eight million L^{-1} to help maintain bio chemical composition, namely lipids, with no significant mortality up to 24 hours and only 5% energy loss. Higher densities can be achieved with no significant increase in mortality (Dhert and Sorgeloos, 1995).

4.4 *Artemia* as Prey

Artemia is one of the most used preys in aquaculture due to its easy culture and manipulation. Since its discovery in 1930, it's widely used in mass culture of commercially important species around the world.

Most species will accept formulated feeds easily as they grow, due to mouth size, development stage of the digestive system and its efficiency (Dhert and Sorgeloos, 1995). As *Artemia* production costs are higher than formulated feeds, cost-effectiveness of *Artemia* must be established in order to perform weaning as early as possible.

Different stages of *Artemia* will differ in biochemical profile, between embryonic form, up to adult. Different strains can also dictate cyst volume and weight, nauplii length, weight, volume and energy content. Choosing *Artemia* starts with selecting a cost-effective strain, followed by most suitable stage.

Brine shrimp can be used in both fresh and seawater as their osmoregulatory system (hypo-osmoregulator) allows them to survive in low salinity environments. In seawater they can survive for several days without food and in fresh water they continue to swim up to 5 hours, when they perish from osmotic stress (Dhert and Sorgeloos, 1995).

Instar I and II are the most used forms of *Artemia*, as they are the easiest form to get from cysts. The non-feeding Instar I will reflect parental characteristics, while Instar II

will reflect diet profile (Dhert and Sorgeloos, 1995). Adults contain high energy content, however they are not commonly used due to necessary labour and infra-structure.

5 *Solea senegalensis* (Kaup, 1858)

This species belongs to Soleidae family, a group of flat fish that have flat and oval shape, presenting an altered bilateral symmetry.

Solea senegalensis (Figure 5) display both eyes on the right side, named ocular side. Dorsal fin starts right after the eyes location and both dorsal and anal fin do not contain spines, but instead soft rays. The mouth is located under the left eye and they possess brown coloration, on the ocular side, with variations from dark to light. Senegalese sole has a high ability to mimicry in order to adapt to the environment (Healey, 1999). Adult specimens usually reach between 45 - 60 cm in length (Abellan and Basurco, 1999).

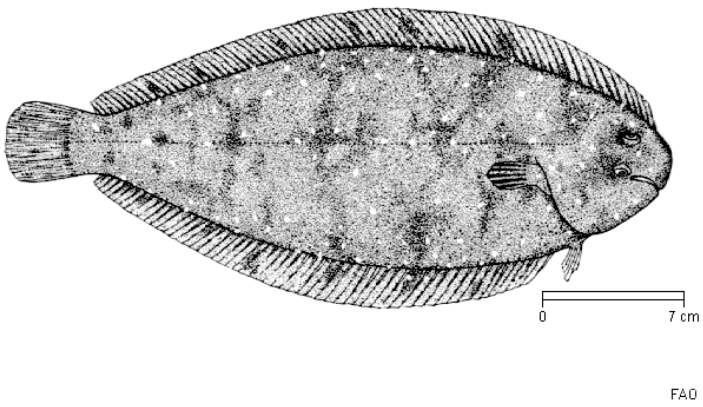

Figure 5 - *Solea senegalensis* (FAO)

Solea senegalensis is euryhaline, capable of adapting to salinities between 30 ppt (Rueda-Jasso *et al.*, 2004) to 38 ppt (Ambrosio *et al.*, 2008; Salas-Leiton *et al.*, 2008). As benthonic species, it can endure low levels of dissolved oxygen (Salas-Leiton *et al.*, 2008) and inhabits sandy or muddy bottoms of coastal and estuarine areas, up to 100 meters in depth, with temperatures range between 13 - 28ºC (Vinagre *et al.*, 2006). It's a sedentary

species, not undergoing long migrations (Walker and Emerson, 1990; Rijnsdorp *et al.*, 1992).

Solea senegalensis feeds on benthonic invertebrates such as polychetes, bivalve molluscs and small crustaceans (Arias and Drake, 1990; Abellan and Basurco, 1999; Cabral, 2000) and age plays a determinant factor in type of prey consumed (Whitehead, 1986; Quéro and Vayne, 1997).

II. Internship at *Solea senegalensis* hatchery Safiestela, S.A.

1 *Solea senegalensis* production

For the last decades European aquaculture industry grew substantially. About 35 aquatic species are produced, dominated by rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), gilthead sea bream (*Sparus aurata*), common carp (*Cyprinus carpio*) and European sea bass (*Dicentrarchus labrax*). According to Dinis *et al.* (1999), these species already show signs of market saturation and *Solea senegalensis* presents a good alternative, being a native species with high market value, whose biological cycle can be reproduced in captivity.

Being well adapted to warm climates this species is commonly raised in extensive earthen ponds along the south coasts of Portugal and Spain (Drake et al., 1984; Rodríguez Martínez, 1984; Dinis, 1986; Dinis, 1992).

Sole wild annual catches oscillate, are hard to predict, and are declining (Imsland *et al.*, 2003), making it an ideal candidate for production. It has already been suggested for a few decades for intensive recirculation systems (Dinis *et al.*, 1987; Dinis, 1992; Vázquez *et al.*, 1994; Marin-Magan *et al.*, 1995; Howell, 1997; Anguis and Canavate, 2005; García and García, 2006; Gavaia *et al.*, 2009; Padrós *et al.*, 2011), as it can achieve high commercial value (12.25 €/kg in MercaMadrid, 2013) and high market demand.

The world production of flatfish increased from 26,300 tons in 2000 to 148,800 tons in 2008, with Spain being the major producer in Europe (FAO, 2015). The sole production in Southern Europe has also increased significantly, with Spain producing 60 tons in 2005 and 194 tons in 2012 and Portugal, 11 tons to 100 tons, respectively.

Advantages concerning rearing of sole include natural spawning in captivity, high egg production, high survival, fast development of eggs and larvae, high growth rate in juveniles and even the possibility of adaptation of existing production facilities to accommodate its rearing. *Solea senegalensis* is also tolerant to variations of environmental rearing conditions, as temperature, oxygen and salinity, and presents a better growth rate than common sole, *Solea solea* (Dinis *et al.*, 1999; Imsland *et al.*, 2003; Howell *et al.*, 2006). This species is popular among consumers and usually with a high market value (Reig *et al.*, 2000), being indistinguishable by consumers from common sole (*Solea solea*, Linnaeus, 1758).

Larval nutrition, weaning protocols, feed technology and on-growing rearing techniques (Howell, 1997; Dinis *et al.*, 1999; Conceição *et al.*, 2007) have been improved to the point of successfully produce individuals under intensive industrial conditions (Imsland *et al.*, 2003; Cañavate, 2005). Advances in recirculation systems made possible to fully control optimal growth conditions for rearing sole all year round.

2 The Company

The internship was carried out for 7 months in the facilities of Safiestela - Sustainable Aqua Farming Investments, S.A., located in north Portugal in Lugar do Rio Alto, Póvoa de Varzim. This company is responsible for the mass production of *Solea senegalensis* juveniles and belongs to the group Sea 8, together with another facility, Aquacria Piscicolas, S.A. located in Torreira, responsible for the ongrowing phase of sole production.

Safiestela is divided in different sections according to this species development stage. Each area contains its own protocols, personnel and equipment, though avoiding cross contamination between different sections of the facility.

2.1 Broodstock

Reproduction in captivity is one of the main bottlenecks of sole domestication (Howell *et al.*, 2011b). It is not fully controlled, as F1 and F2 individuals are incapable of producing fertile spawning, with commercial hatcheries still relying on wild broodstocks (Cañavate, 2005). Commercial production requires a good management of its broodstock, since the loss of genetic variability can lead to negative effects in performance traits, such as growth (Falconer, 1960). This is a major problem in the production cycle, as accurate genetic knowledge of wild broodstock is difficult to attain. In addition, throughout the breeding season, some individuals tend to breed more than others (Porta *et al.*, 2006), decreasing genetic variability.

The problem related to F1 individuals is not well understood, but some studies suggest its poor spawning is not related to any hormonal dysfunctions (Bertotto *et al.*, 2006; Agulleiro, 2007; Guzmán *et al.*, 2008).

F1 females present normal vitelogenin, steroid profiles and spontaneous spawning (Guzmán *et al.*, 2008), with egg quality parameters within normal range (Guzmán *et al.*, 2009). However these eggs show no fertilization in the broodstock rearing tanks when paired with F1 males, even though F1 females are still able to produce fertile eggs when paired with wild males (Mañanos, 2011). This suggests that the problem might be related to the F1 males, which seem to display a decreased efficiency in sperm production and sperm functionality when compared to wild males (Forne *et al.*, 2011). Nonetheless, the major concern appears to be the F1 males courtship abnormal behaviour when compared to wild males (Carazo *et al.*, 2009; Carazo *et al.*, 2011), where males swim underneath a female, with synchronized movements, towards the surface, behaviour similar to the one observed in the mating of the common sole (Baynes *et al.*, 1994).

Other factors such as rearing conditions in larval stages, the genetic composition of broodstock or even broodstock nutrition could also be the motive for this problem (Bromage and Roberts, 1995; Mañanós *et al.*, 2008; Howell *et al.*, 2011a; Norambuena, 2012).

Solea senegalensis is a gonochoric species and presents no external dimorphism. Females mature at the age of 3 years with a total length of 32 cm (Dinis, 1986) and maturation can be verified through visual inspection of the abdominal region for swelling, or by evaluation of condition index (Anguis and Canavate, 2005).

Safiestela receives it's broodstock from captured wild specimens. These individuals are acclimated through a quarantine period and blood samples are taken to determine sex and test for any disease or virus. Every fish is then chipped to track their development and condition index.

Temperature is a major factor in sole reproduction, as natural spawning takes place from March to July (Dinis, 1986; Andrade, 1990; Dinis *et al.*, 1999) and in Autumn, with temperatures between 13 - 23ºC, achieving maximum fecundity around 15 - 21ºC (Anguis and Canavate, 2005). To achieve all year round production of eggs, Safiestela's broodstock area is divided into four different sections with controlled temperature and photoperiod according to seasons (Spring, Summer, Autumn and Winter). Each breeding group is prepared to spawn in the designated room season (Spring room – spawn during

Spring), and natural spawning is induced by increasing temperature in the tanks up to 2.5ºC within 3 days (Anguis and Canavate, 2005).

Each room (Figure 6) is equipped with 3 - 5 tanks with low density (1 - 1.5 kg / m^2) and 33 - 35 ppm (Imsland *et al.*, 2003), running in independent recirculation systems. Each breeding group is kept with a higher proportion of males to females, to ensure good fertilization. Each female can place between 100,000 to 150,000 eggs/kg and fertilization rate is around 44.9 ± 18 to 86 ± 14.2% (Dinis *et al.*, 1999; Anguis and Canavate, 2005). Eggs diameter range between 0.87 – 1.0 mm, are planktonic and have a golden colour (Lagardère, 1979; Rodríguez Martínez, 1984; Dinis, 1986). Egg size may decline along the spawning period (Dinis *et al.*, 1999; Anguis and Canavate, 2005).

Figure 6 – Wild Broodstock tanks in Safiestela

Eggs float, so tanks are equipped with egg collectors at water outlets, with aeration and 400 µm nets. Spawning synchronize with dusk periods, taking off after dusk and peaking about 4 hours later. It synchronizes with lunar phases as well, peaking in new moon (Howell, 2009). During spawning, floating eggs are trapped in these collectors and are collected in the morning. Eggs are then weighted, tagged and then transferred to the incubation tanks.

2.2 Incubation Room

Incubation time can range, depending on rearing conditions, between 36 hours at 20ºC to 48 hours at 17ºC (Dinis and Reis, 1995; Canavate and Fernández-Dıaz, 1999; Dinis *et al.*, 1999). Temperature may be connected to sex differentiation in sole breeding, as daily thermocycles can determine sex ratios during larval rearing (Blanco-Vives *et al.*, 2011). During this stage, free amino acids are the most important energy substrate for the embryo (86%) (Parra *et al.*, 1999).

The incubation room (Figure 7) is equipped with 100 L conical tanks operating in open system and incubation is performed in total darkness with 1000 – 10,000 eggs L^{-1} (Dinis *et al.*, 2007). Tanks are kept with slight aeration and slow water renewal until hatching (Imsland *et al.*, 2003; Conceição *et al.*, 2007).

Figure 7 – Incubation room of Safiestela

Planktonic larvae emerge with bilateral symmetry and depending on egg quality and size, measuring around 2.4 ± 0.1 mm in total length (Dinis *et al.*, 1999), usually larger eggs produce larger larvae (Dinis, 1986; Bedoui, 1997; Geffen *et al.*, 2007).

During incubation, samples are taken to estimate hatching rate and the total number of larvae in each tank.

Larvae stay in this room until 1 day after hatching (DAH), when they are transferred to the larvae rearing room.

2.3 Larvae Room

Two days after hatching (2 DAH) sole larvae reach 3 - 3.3 mm in length and mouth and anus become functional (Ribeiro *et al.*, 1999a), a critical moment in larvae development with the start of exogenous feeding (Dinis *et al.*, 1999), with only 5% of the yolk reserves remaining (Parra and Yúfera, 2001).

With a mouth gap of 350 µm (Parra and Yúfera, 2001), sole larvae can feed on *Artemia* nauplii as first prey (Magalhães and Dinis, 1996), but larval protocols include feeding with rotifers in the first days, to allow HUFA enrichment (Dinis *et al.*, 1999). Feeding protocols usually follow a use of rotifers for the first 10 days after hatching and co-feeding with *Artemia* from day 4 - 5, followed by the use of *Artemia* until weaning (Dinis, 1992; Canavate and Fernández-Dıaz, 1999; Martinez *et al.*, 1999).

Stomach and gut are not fully developed until metamorphosis (Ribeiro *et al.*, 1999b). Thus, digestion in pre-metamorphic larvae is not acidic and depends on pancreatic enzymes (Ribeiro *et al.*, 1999b) and pinocytosis in the rectal epithelium. Larvae digestive capacity will increase with age in later stages (Rust, 1995).

Fish larvae have high metabolic demands due to high growth rates (Houde, 1997), and it is believed that high frequency feeding maximizes growth in fish (Haylor, 1993). As result, commercial hatcheries supply several meals per day to larval rearing tanks or adopt a continuous food delivery system. These systems assure high performance in larvae growth, decreasing size variation and ultimately reducing size dispersion on lots (Chen and Purser, 2001).

This room (Figure 8) is equipped with several conical 3 m^3 tanks running in an open system with gentle aeration and water renovation. Water is previously filtered and sterilized with UV treatment. Salinity is kept around 35 ppm, oxygen at 90 - 100% saturation and temperatures between 16 - 23ºC (Dinis *et al.*, 1999). Density is kept around 13 larvae L^{-1}.

Figure 8 – Larvae rearing room in Safiestela

Water quality is sampled every 4 hours with a multiparametric probe to ensure that conditions remain constant to avoid larvae stress. *Solea senegalensis* can adjust its osmoregulatory system to compensate the effects of temperature on electrolyte transport capacity and thyroid hormones implicated in temperature acclimation (Arjona *et al.*, 2010). Nonetheless sole larvae are very sensitive to water temperatures variation and should be avoided, as they can irreversibly affect fish grow performance (Johnston and Hall, 2004).

Pre-metamorphic larvae depend on light to capture live prey, as their visual range, for most fish larvae, is at best two body lengths (Wahl *et al.*, 1993), making live prey small and low contrast targets, extremely difficult to detect. Every tank is equipped with its independent light system. Light intensity need to be at least 1,200 lux at the surface and photoperiod can be of 16L : 8D or continuous light (Conceição *et al.*, 2007). During the first rearing days, green water method is used in larvae tanks with *Isochrysis galbana*, *Tetraselmis* sp. or *Nannochloropsis sp.* This procedure helps stabilizing the water quality on rearing tanks, serves as a food source for live zooplankton, increasing its nutritional value, and provides better prey contrast for sole larvae, better light dispersion and assists with microbial control.

Metamorphosis describes a rapid morphological and physiological change which follows a stable period of slow growth in fish early stages. Usually lasts for a week and starts around 10 days after hatching, achieving its apex around 15 - 16 days at 18 - 19ºC (Ribeiro *et al.*, 1999a; Fernández-Díaz *et al.*, 2001), but its duration is also related to food

availability and type (Fernández-Díaz *et al.*, 2001). At the end of metamorphosis fish larvae measure around 7.3 ± 0.8 mm (Imsland *et al.*, 2003). During metamorphosis fish settle in the bottom of their rearing tanks, changing their feeding habits, behaviour and ecologic niche.

During metamorphosis larvae lose their bilateral symmetry, as their left eye undergoes a 90º migration into the ocular side (Figure 9), and their bodies begin to flatten assuming an asymmetrical pigmentation (Fernández-Díaz *et al.*, 2001). The success of this process depends on larval size, energy reserves and feeding capacity during metamorphosis (Geffen *et al.*, 2007).

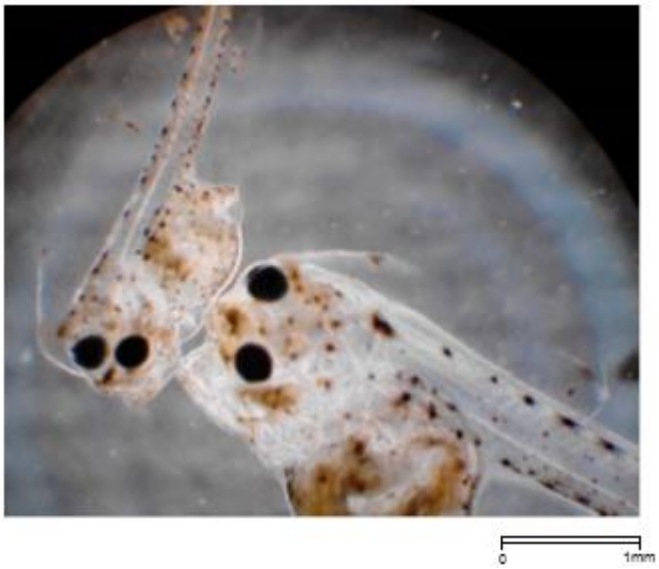

Figure 9 – *Solea senegalensis* larvae at 12 DAH. Photograph taken by Isidro Blanquet.

During this process growth declines, because of reduced feeding and due to the energetic demanding changes undergoing in fish body (Yúfera *et al.*, 1999; Fernández-Díaz *et al.*, 2001; Cañavate *et al.*, 2006). These include modifications to fish digestive system, as it changes from an undifferentiated tube to a system with a stomach, liver, pancreas and intestine.

Fish settling in the bottom of the tanks is an indicator of metamorphosis stage and marks the point where post-metamorphic larvae prefer to graze continuously on *Artemia* in

the bottom of the tanks (Dinis *et al.*, 2000). As a consequence, larvae are transferred from these tanks to the weaning area.

2.4 Rotifer Room

The best system to implant in a commercial hatchery greatly depends on production scale objective, seasonally or continuous production of larvae, reliability of the culture and staff experience. Reliability is crucial as it dictates the number of tank replicates necessary to achieve the production goal, by safeguarding a minimum production in case of culture collapse.

The production cost is dependent on culture scale, with production costs of 0.036 € per million rotifers in a system producing 4 billion per day, and 0.134 € per million rotifers in a 1 billion per day production system (Dhert and Sorgeloos, 1995).

Safiestela uses a type 4 batch culture system for *Brachionus plicatilis* (Figure 10) rotifer production. Batch cultures are closed systems set up for high production with controlled set of conditions in order to optimize growth. These are indoor mass production systems with short production cycles (3 days) in 1 m^3 cylindrical tanks that start with inoculation of 10,000 rotifers mL^{-1} and can reach, after 3 days, up to 20,000 – 30,000 individuals mL^{-1} (Dhert and Sorgeloos, 1995). Due to the high densities, oxygen gas has to be supplied to prevent dead zones in the tanks and hydrochloric acid is added to the system to stabilize the pH at 7. This prevents pH variations during the production cycle, caused by the accumulating waste products.

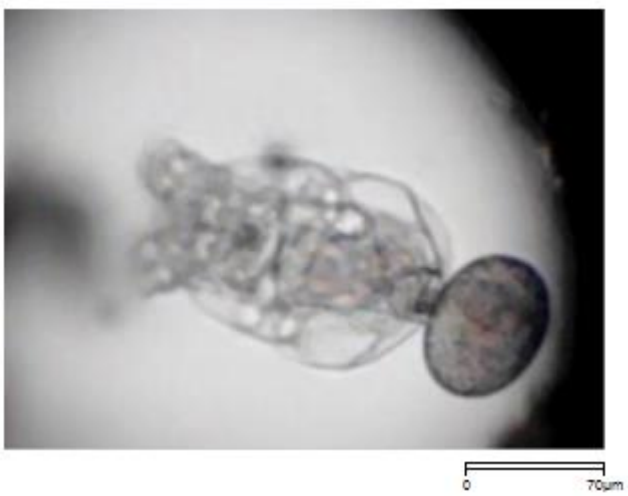

Figure 10 – Rotifer *Brachionus plicatilis*. Photograph taken by Isidro Blanquet.

Three to four culture tanks are maintained in this room (Figure 11) and after 3 days of culture, each tank is harvested for further enrichment prior to feeding the rotifers to the larvae. A portion of the harvested rotifers are used to start a new culture. The three tanks ensure that every day a different tank is ready for harvest, achieving a constant production of rotifers during larvae rearing periods.

Figure 11 – Rotifer room in Safiestela

Tanks are kept under permanent light photoperiod (24 hour light) and pure oxygen is supplied, with oxygen saturation between 80 - 90%. Air is also injected in the bottom of

the tanks to help recirculation. Salinity is kept around 35 ppm and temperature is maintained at 28ºC by industrial water heaters.

Rotifers are feed daily with a mixture of algae and yeast. Due to the culture system used, production starts with total tank volume and low rotifer densities. This poses a problem, as in the first days of culture rotifers are not able to fully filter culture medium, to clear the water. Food waste can quickly deteriorate water quality and lead to bacterial growth, so in order to avoid these problems, rotifers meals are divided in 6 portions per day, to keep a better water quality and provide a more stable environment, especially in the first days.

On the third day (Figure 12), culture is filtered using a concentrator to help separate rotifers from the culture waste, and then rinsed in freshwater to remove any possible ciliates and pathogens. Rotifers are then enriched with essential fatty acids and proteins with a commercial emulsion for 24 hours before feeding them to the larvae. After enrichment rotifers are kept in a cold storage container to help reduce metabolism losses and maintain the fatty acid profile.

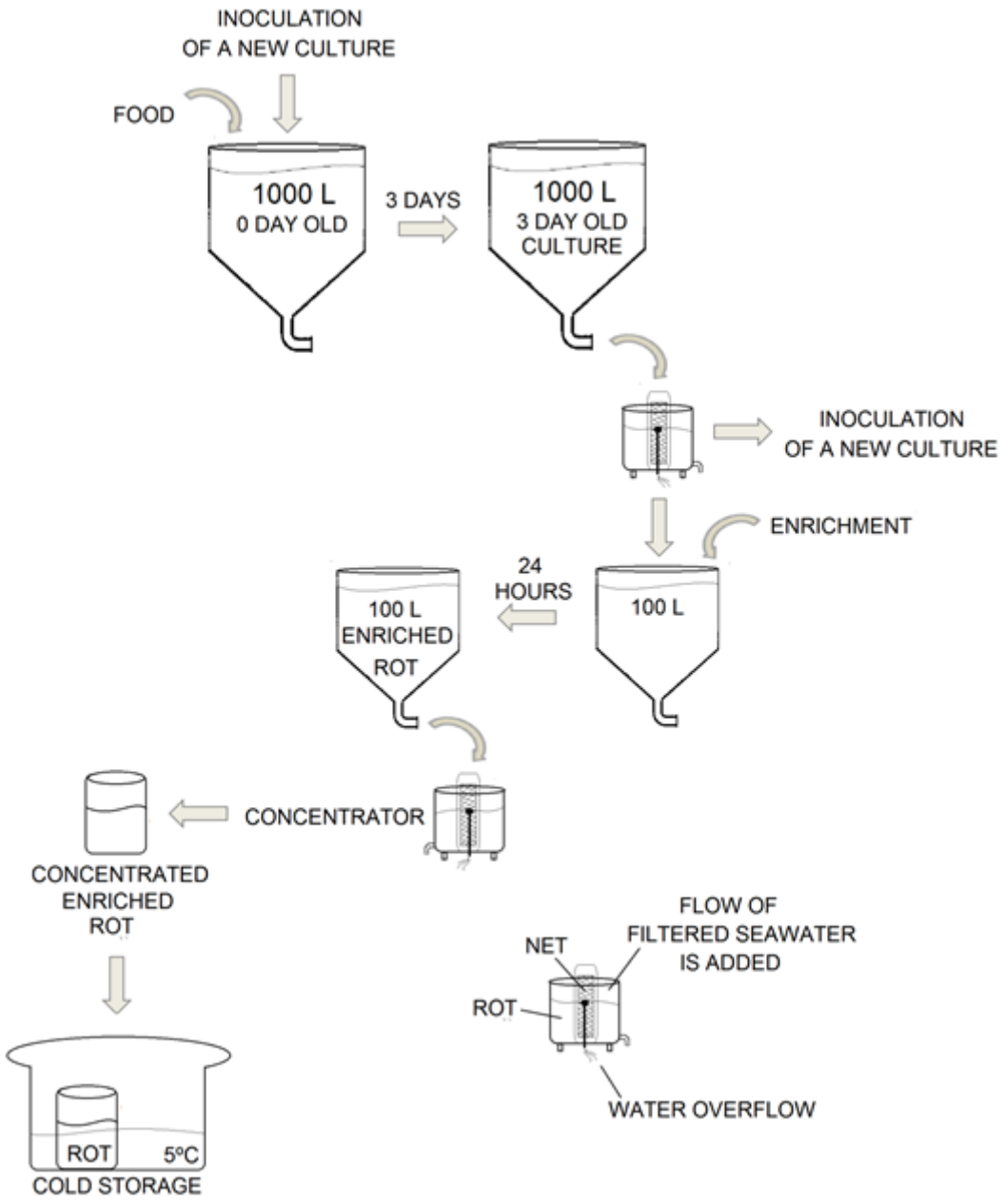

Figure 12 – Rotifer cultures maintenance running in batch system (3 day cycle) in Safiestela

2.5 *Artemia* Room

Artemia production is divided into two stages, hatching and enrichment. The room is equipped with 100 L cylindrical-conical tanks for hatching and 1000 L tanks for enrichment. Each step takes 24 hours, so it's up to the person responsible for the live feed

production to prepare the right amount of *Artemia* require for sole feeding, two days in advance.

Artemia cysts are placed in a hatching tank with continuous light and 28ºC filtered seawater. The next day, hatched *Artemia* tank can be filtered and enriched for 24 hours in the enrichment tanks. These are prepared with filtered seawater at 28ºC and continuous light conditions, pure oxygen and air supply, to help support high densities and good water mixing. Commercial enrichment is supplied to the culture 6 times in the next 24 hours.

After enrichment *Artemia* (Figure 13) is ready to be filtered, using a concentrator, and then washed in filtered seawater to remove any suspended solids or pathogens that could affect the fish tanks. Both hatching and enrichment tanks are sampled daily to estimate total population.

Figure 13 – *Artemia sp.* AF nauplii. Photograph taken by Luís Calisto.

Enriched *Artemia* is then placed in cold storage equipment at 5ºC in order to reduce fatty acid profile losses due to inherent *Artemia* metabolism. Maintenance of *Artemia* production is resumed in figure 14.

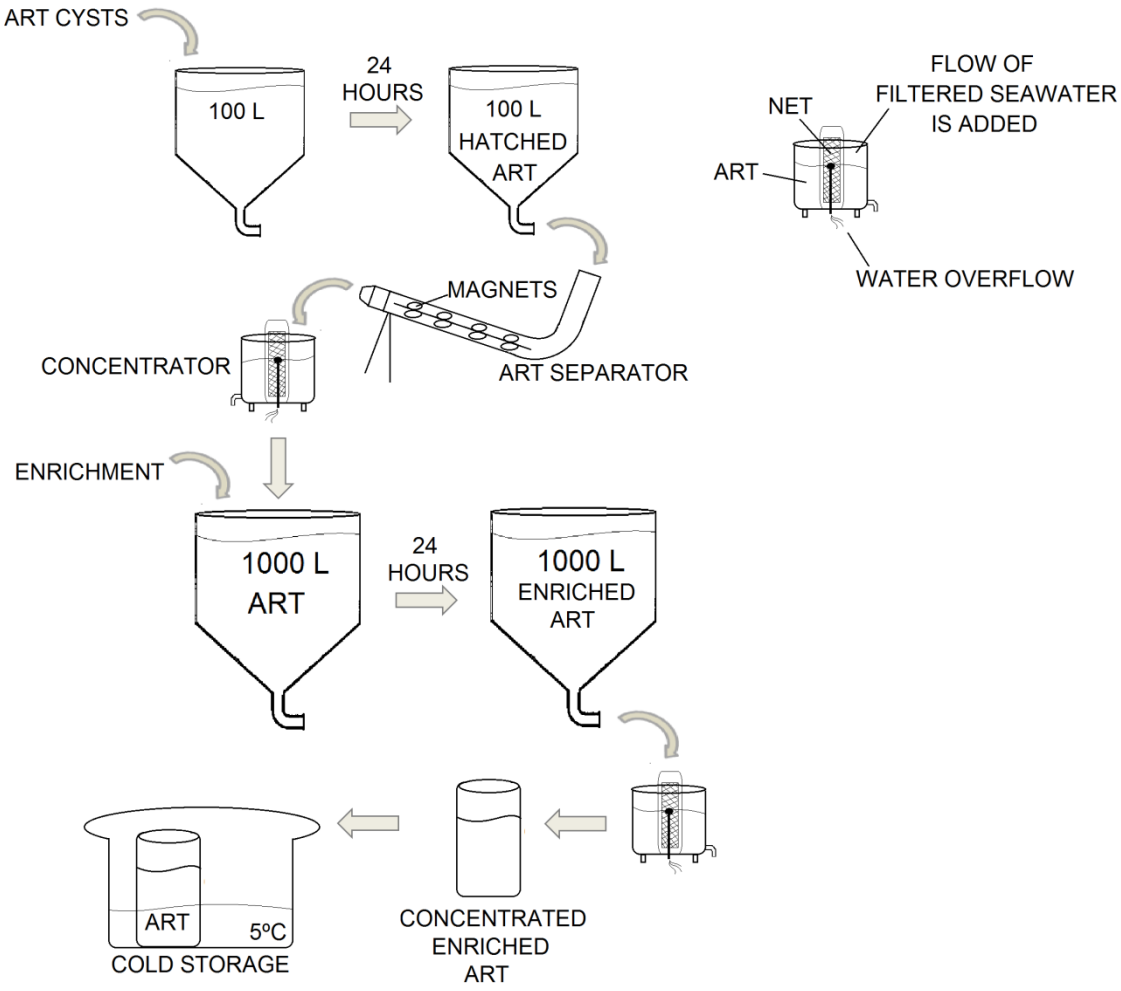

Figure 14 - *Artemia* production in Safiestela

2.6 Weaning

Weaning is another critical moment in the production of *Solea senegalensis*. During this period the feeding of fish change from live prey to inert diets. This step must be carefully planned, as mortality can be high in small postlarvae (Ribeiro *et al.*, 2002).

Solea senegalensis weaning is more difficult than in other marine species, because of the feeding behaviour of post-metamorphosis larvae (Howell, 1997; Dinis *et al.*, 1999). Basically two strategies can be chosen, abrupt weaning or co-feeding with both *Artemia* and inert diets, implementing a gradual substitution of *Artemia* (Day *et al.*, 1997).

Weaning was proposed to be performed at 30 - 40 DAH (Canavate and Fernández-Dıaz, 1999; Ribeiro *et al.*, 2002), coinciding with the formation of gastric glands, 27 - 31 DAH (Ribeiro *et al.*, 1999a) and thus achieving acid digestion with a complete digestive tract (Ribeiro *et al.*, 1999b). However post-larvae size is related to the maturation of the digestive tract, so weight can be used as an indicator of larvae development stage and physiology to help determine the weaning strategy (Verreth, 1994; Rosenlund *et al.*, 1997; Engrola *et al.*, 2007).

Fish settlement in the bottom of the larvae tanks signals the end of larvae rearing period. Larval tanks are emptied and all larvae are transferred to the weaning area as post-larvae niche and feeding behaviour changes from pelagic to dwelling stage, requiring less water column.

The weaning area (Figure 15) in Safiestela is divided in two regions, each is equipped with square tanks with 20 cm water column to facilitate cleaning and fish monitoring. Weaning 1 tanks are designed for post-larvae, while they still eat live feeds and run in an open-flow system with previously filtered and heated sea water. Weaning 2 system is designated for weaned fish, that already eat formulated feeds and these tanks run in a closed system.

Figure 15 – Weaning room in Safiestela. Photograph taken by Luís Baião.

Until 30 DAH fish are fed manually with live prey and then changed to formulated feeds, distributed by an automatic feeder. Meals are distributed through the day in order to decrease food retention times and increase prey and water quality.

Temperature is kept around 20ºC and salinity at 35 ppm, with higher temperatures increasing the risk of pathologies (Howell *et al.*, 2011a). These parameters are checked every 4 hours by the technicians assigned to this area. Daily water quality tests include nitrogen compounds, oxygen levels, water turbidity and system redox potential.

2.7 Pre-fattening

When fish reach 1 g they are screened and then transferred to the pre-fattening area to be reared until 40 g, when they are ready to be transferred to the fattening unit. Aquacria is then responsible to rear the fish up to commercial size.

Solea senegalensis can be reared in shallow fiberglass raceway tanks and fed with inert feeds in a controlled production environment (Imsland *et al.*, 2003). Temperatures should be kept under 25ºC in order to decrease risk of pathologies (Palazzi *et al.*, 2006), following natural thermoperiod or kept at 20ºC (Ambrosio *et al.*, 2008; Salas-Leiton *et al.*, 2008; Borges *et al.*, 2009; Costas *et al.*, 2011).

In Safiestela, pre-fattening area (Figure 16) is kept in low light conditions with blue lights. It's equipped with 50 raceway tanks (12 x 2 m), with low water column, running in a closed system. Recirculation is maintained with the use of a RAS system, in order to reduce water use, while keeping an efficient removal of nitrogen compounds and overall waste.

Figure 16 – Pre-fattening area in Safiestela. Photograph taken by Isidro Blanquet.

2.8 Safiestela daily tasks

The working schedule at Safiestela, S.A. is from 8 am – 5 pm and technicians are assigned to different areas, each one with its own routines and challenges.

The **broodstock room** is generally controlled by one technician and most of its tasks take place in the morning.

The morning starts with the inspection of all egg collectors in this area. In case of presence of eggs, they are carefully collected to avoid any physical damage and weighted. Before transferring them to the incubation room, a viability test is performed, by examining egg buoyancy in a 30 - 35 ppm water container, fertile eggs will float and non-fertilized eggs will sink. Each batch of eggs is then tagged to its breeding group and transferred to the incubation tanks. At the end, all egg collectors nets are cleaned and disinfected.

The next task is to prepare the broodstock pellets and feed them manually, taking advantage of this time to evaluate adult sole condition and behaviour and also monitoring the amount of food given, thus avoiding any food surplus in the tanks. During feeding water parameters are also determined.

After feeding all tasks in the morning are completed and the technician assigned to this area is free to go help other stations, usually weaning room.

Nonetheless, water temperature and oxygen are checked thorough the day, and water samples are taken to determine nitrogen compounds levels.

Periodically adult fish are analysed for measurement and weight to evaluate condition index and maturation stage. This is generally done by 2 technicians due to the fish size.

In the afternoon sole tanks are cleaned to remove any waste, followed by cleaning and disinfection of all equipment, floors and footbaths in this area.

At the end of the working shift egg collectors are cleaned and disinfected and prepared for the next night.

The **larvae room** is kept by one technician. Before the new production cycle begins, all equipment, tanks and nets are disassembled and disinfected and nets from the water outlets in the tanks are replaced. The entire room is also cleaned and disinfected, including floor, walls and ceilings.

In the morning, this area is the most important to attend to, as larvae need to feed at 9 am.

The first task is to adjust algae concentration in the tanks, followed by the sampling of the tanks to determine the prey concentration before feeding the larvae. Adjustments are made according to the company protocol. Live preys are then distributed by hand.

After the feeding, the technician is free to sample larvae from each tank and determine their length, metamorphic stage and stomach content, as the absence of food can be an early sign concern.

The remaining tasks of this room are monitoring the water parameters of the larvae tanks throughout the day and the larvae feeding, which is done several times per day.

The technician assigned to this room is also responsible for the maintenance of the incubation room.

The **live feed rooms** are the responsibility of only one technician. So in order to quickly prepare *Artemia* and rotifers for sole feeding in the morning, both rooms have to be attended at the same time. As a result most tasks of this area have to be completed in the morning.

Tasks begins with filtering the rotifers and *Artemia* enrichment tanks as soon as possible to feed sole larvae until 9 am. With the live food ready and placed in the cold storage, technicians from the larvae or weaning area can finally attend to the larvae.

In the rotifer room the next task is to prepare the 3 day old rotifer culture to be filtered. The total process takes a long time, as water flow rate must be slow to avoid clogging the nets in the concentrator. When completed, the rotifers are rinsed in filtered seawater and transferred to the enrichment tanks, and a portion of the cultured is used to inoculate a new tank (0 day old).

In the *Artemia* room at the same time the hatching tanks set up in the day before are also filtered using a magnetic separator. This step is also slow, as too much water flow from the tanks can fail to provide a good separation of the *Artemia* nauplii from their hatching waste. *Artemia* is then rinsed in filtered seawater and transferred to the enrichment tanks.

All tanks are cleaned and disinfected in both rooms and prepared to receive a new inoculation.

Rotifer cultures are sampled to estimate population growth and egg ratio, as it can help predict the state of the culture for the next hours. The number of rotifers in 1 mL samples is determined, and the number of females carrying eggs is noted. Egg ratio is estimated by the formula:

$$\mathbf{r = 1/T \ ln \ (N_t - N_0)}$$

Where, T = duration of culture in days; N_0 = initial number of rotifers and eggs; N_t = total number of rotifers and eggs after T days of culture. The r values for this species vary between 0.23 - 1.15. Three different 1 mL samples should be examined to get a more reliable estimate of total population. If variation between them is above 10%, more samples should be examined (Dhert and Sorgeloos, 1995). Total rotifer population will dictate the amount of food per tank.

Both *Artemia* enrichment and rotifer food are prepared and tagged for 6 different meals for the day.

All used tanks and equipment are cleaned and disinfected.

In the *Artemia* room, for the next day, *Artemia* cysts are weighted and placed in the hatching tanks.

At the end of the morning most tasks of this area are concluded and the technician is free to go help other stations in the afternoon. The only remaining tasks are feeding rotifers and *Artemia* and monitor the water quality in both rooms during the day.

The **weaning room** is one of the most labour intensive rooms in Safiestela with over 50 tanks and requires a team of 2 or 3 technicians to operate.

The feeding is performed by an automatic distributor, so technicians can focus on the rearing tanks maintenance.

In the morning all tanks, food distributors exit, water inlets and outlets need to be carefully cleaned to remove any organic waste.

Throughout the day water parameters are checked every 4 hours by the technicians assigned to this area. Daily water quality tests include nitrogen compounds, oxygen levels, water turbidity and system redox potential.

After all cleaning is completed, one technician proceeds to inspect each tank to detect any dead or diseased individuals that need to be removed to reduce the chance of spreading diseases.

The other two technicians start the daily screenings of this area, meant to keep homogenous size in tanks population. These screenings are performed manually and during the procedure, a careful inspection of the fish is performed, where any diseased, under grown, malformed or mal pigmented fish are discarded from production.

In the afternoon, samplings of the tanks are performed to update average weight and correct food quantity and pellet size. The afternoon tasks continue with more manually screenings.

Before the end of the working shift a new inspection to all tanks and a correction of the water inflow rate of every tank is done (according to the company protocol).

The last tasks of the day include cleaning and disinfection of the area floor and footbaths.

The **pre-fattening room** is composed by fifty 12 m raceway tanks running in a closed system. As a result, this is the most intensive labour room, requiring a team of 3 - 4 technicians to operate.

Feeding is done several times a day by an automatic dispenser, like the weaning room, so the technicians of this area can focus on tank maintenance.

In the morning, daily tasks begin with all tanks bottom cleaning, together with all water inlets and outlets to remove any organic waste.

After cleaning, tanks are inspected to remove any possible dead or sick individuals.

After cleaning of the tanks, a group of 2 - 3 technicians is assigned to the size screenings with the help of a grading machine. These are performed daily and require a minimum of two technicians simultaneously. One is responsible for operating the machine, by manually inserting fish, after a fast inspection, in the machine's conveyor belt. A second is required to concentrate the fish in the tanks and manually transport them to the grading machine tank. Fish are graded into three predefined size classes, and are transferred to different new tanks. In order to reduce stress in the fish, a third technician can be used to quickly help transfer fish to the new tanks after screening.

During screenings the rest of the technicians in this area disassemble, clean and disinfect every emptied tank and its equipments and prepare them to receive new fish.

Inspections of the water temperature, salinity and nitrogen compounds levels are controlled at least twice a day. Redox potential and water turbidity are also measured to analyse the biological filter performance.

During the afternoon the size grading continues. In the absence of gradings, the team is free to attend to the room, cleaning all tank structures, walls and columns.

Afternoon tasks also include tanks sampling to update size and weight averages and correct food amount or/and pellet size according to the fish growth and to manage future screenings.

The last tasks of the day include an inspection to the RAS system, specially the skimmer and the ozone generator levels.

A final inspection to the water inflow rate of every tank is performed before the end of the day. These are regulated according to the tank density and fish weight (according to the company protocol).

The floor, footbaths and all equipment like nets and cleaning apparatus are disinfected.

Once per month fish are transferred to the on-growing facility of Aquacria, this is an enormous operation in Safiestela, where personnel from each area is assigned to help. In this procedure fish are collected from the pre-fattening rearing tanks, weighted and transferred to the transportation tanks in a truck. This step is carefully supervised by the Safiestela director, to ensure a good distribution of the fish in the transportation tanks. This transport occurs twice a day (morning and in the afternoon), and can last for 3 days straight. Usually this transportation of fish from Safiestela is followed by a transfer of 1 g fish from the weaning area to the pre-fattening room.

3. Sole Quality

Skeleton deformities in teleosts aquaculture is a common problem worldwide, being responsible for high economic losses, biological performance and animal welfare concerns.

Affected individuals can be labour and cost intensive to industrial productions, as they must be manually screened from production repeatedly. In Europe, aquaculture losses for fish abnormalities incidence are estimated to reach 50,000,000 euros per year (Haga *et al.*, 2011).

Larval quality in reared *Solea senegalensis* (Figure 17) is a major bottleneck in commercial hatcheries, as the presence of morpho-anatomical abnormalities under intensive rearing conditions affects up to 44 - 80% of cultured sole (Gavaia *et al.*, 2002; Engrola *et al.*, 2009b; Gavaia *et al.*, 2009; Fernández and Gisbert, 2011), contrary to low incidence found in wild individuals (Gavaia *et al.*, 2009). These abnormalities, that affect internal anatomy, can be reflected in fish external morphology, together with pigmentation disorders in reared fish, leading to severe economic losses (Koumoundouros, 2010).

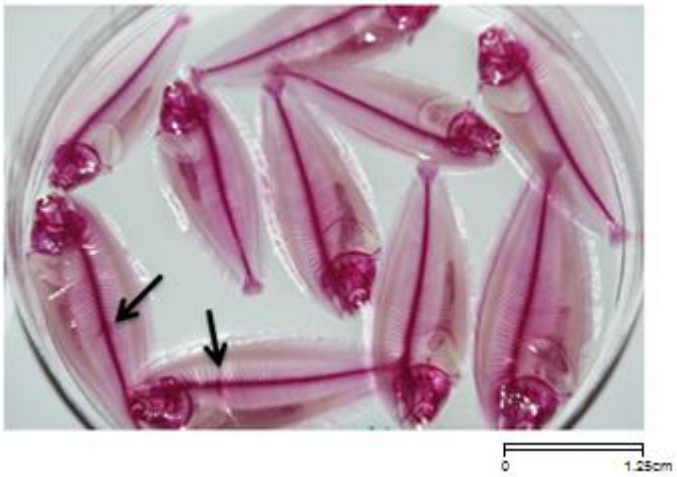

Figure 17 - *Solea senegalensis* juveniles at 79 DAH from Safiestela processed with the double staining method described by Gavaia *et al.* (2000), which uses Alcian Blue 8GX and Alizarin Red S, for cartilage and bone staining respectively. Arrows indicate vertebrae fusions in sole column.

Skeleton deformities can have a negative impact on market value and fish welfare, as it downgrades the image of aquaculture products and can negatively affect fish performance in growth rate, survival and resistance to diseases (Divanach *et al.*, 1996; Koumoundouros *et al.*, 1997; Boglione *et al.*, 2001; Gavaia *et al.*, 2002; Cahu *et al.*, 2003; Koumoundouros, 2010). The causes are still poorly understood, although it has been suggested to be mainly induced in embryonic and larval stages.

Skeleton deformities are classified into three main body regions: cranial deformities, vertebral deformities and fin deformities (Koumoundouros, 2010). They consist in multiplication or absence of bone structures and modifications of position or shape.

Most common deformities in *Solea senegalensis* affect the pleural vertebrae and caudal fin regions (Gavaia *et al.*, 2002; Engrola *et al.*, 2009b; Fernández *et al.*, 2009; Gavaia *et al.*, 2009; Fernández and Gisbert, 2011). Common malformations include vertebrae fusion and anomalies and abnormal vertebral arches (Engrola *et al.*, 2009a; Fernández *et al.*, 2009; Cardeira *et al.*, 2012).

Cranial deformities can severely affect fish welfare and performance as they can negatively impact visual, sensorial, feeding and respiratory functions (Koumoundouros, 2010). In Senegalese sole, cranial deformities include problems with eye migration during metamorphosis, reaching up to 5% of reared individuals (Gavaia *et al.*, 2009).

Vertebral deformities affect fish column with heavy impacts on fish performance and external physiology, as they disturb the axis responsible for the support of musculature for swimming (Koumoundouros, 2010). They can reduce fish size and change its shape (i.e. circular shaped fish). They can modify fish vertebra and induce curvatures like lordosis, V shaped dorsal-ventral, kyphosis, Λ shaped dorsal-ventral curvature, and scoliosis, displaying a lateral curvature. These modifications are common in reared sole (Hall, 2005). Vertebral anomalies generally occur during the segmentation of the nothocord and differentiation of vertebral structures (Koumoundouros, 2010).

Fin erosion has been used as a quality indicator for sole, as they can affect external morphology. Fish reared in intensive conditions can display signs of erosion or bitten fins (Noble *et al.*, 2012; Boglione *et al.*, 2013a; Boglione *et al.*, 2013b). Less severe deformities include malformations in fin bony structures: epural, hypurals, parahypural, urostyle, neural and hemal spines (Gavaia *et al.*, 2002; Engrola *et al.*, 2009a; Fernández *et al.*, 2009). However these usually don't affect the external appearance of the fish (Boglione *et al.*, 2013b). Caudal fin anomalies generally occur during the development of posterior notochord (Koumoundouros, 2010).

Solea Senegalensis presents beige colour in the ocular side, although in commercial productions darker or lighter variations can occur (Ruane *et al.*, 2005). Reared sole is commonly affected by pigmentation anomalies, up to 61% in flatfish aquaculture (Estevez and Kanazawa, 1995; Estevez *et al.*, 1999; Copeman *et al.*, 2002; Villalta *et al.*, 2005a). These affect production as they decline product value, as most cases must be discarded from production (Næss and Lie, 1998; Bolker and Hill, 2000). These disorders occur at the metamorphosis stage, when chromatophores undergo differentiations.

III. The effects of the substitution of Instar I *Artemia sp.* AF by enriched Instar II *Artemia sp.* EG in the first days of *Solea senegalensis rearing*

1 Aim of this trial

The objective of this trial was to improve sole larvae rearing protocols in the commercial hatchery Sea8 in Portugal.

The trial compared the effects of the company's feeding protocol in reared *Solea senegalensis* larvae, which includes the use of Instar I *Artemia sp.* from AF strain (Inve, Aquaculture) for 5 days prior to the use of enriched Instar II EG strain (Inve, Aquaculture), for the earlier use of enriched Instar II EG strain *Artemia sp.* (Inve, Aquaculture) after rotifer feeding period in the early stages of sole rearing.

AF *Artemia sp.* is a small nauplii strain, around 480 µm, ideal for newly hatched fish larvae. It possesses high HUFA level content at hatching without needing further enrichment (Inve, Aquaculture). This strain is easy to use, as it can be delivered to small larvae as nauplii without secondary procedures.

EG strain is a common on growth type of *Artemia sp.* with low HUFA content that needs enrichment before feeding it to larvae. These *Artemia* display a bigger size and volume when feeding it to larvae due to the 24 hour enrichment procedure required.

When compared to EG strain, AF is a smaller type of *Artemia* ideal for small larvae early feeding. It induces a good feeding response from sole due to its bright colour and it can be offered to larvae right after hatching. However this strain has a higher cost when compared to the EG strain.

Hence the objective of this trial was to test if the early substitution of *Artemia sp.* AF nauplii for the bigger and less expensive enriched EG *Artemia sp.* would compromise the normal development of sole larvae, due to its bigger size.

If successful, Safiestela, S.A. can modify their feeding protocols by not using two different types of *Artemia* during sole larvae rearing, and using only 2 live organisms, rotifers and *Artemia sp.* EG. This could simplify the live feed production protocols in the company, with only one hatching and harvesting of one type of Artemia per day. With the increasing benefits of replacing the use of a higher cost/kg *Artemia sp.* AF for 5 days in sole rearing for an early introduction of a lower cost/kg *Artemia sp.* EG strain.

2 Material and Methods

2.1 Larval Rearing

This experiment was performed under commercial industrial rearing conditions, in order to evaluate the results from the substitution of Instar I *Artemia sp.* AF with earlier co-feeding of enriched Instar II *Artemia sp.* EG after rotifers feeding phase in standard *Solea senegalensis* feeding regimes.

Solea senegalensis eggs were obtained from natural spawning of broodstock held under controlled photo and thermo conditions in SAFIESTELA, S.A. (Póvoa de Varzim, Portugal). These were then incubated at 19ºC for 4 days in conical 100 L incubation tanks.

For the experiment, four cylindrical tanks of 3 m^3 running in an open system were used. Larvae were produced from the same breeding group and were randomly distributed by these tanks, with 35,000 larvae (approximately 13 larvae L^{-1}). Temperature was kept at 20ºC and salinity 35 ppm. A 16L : 8D photoperiod cycle was maintained with light intensity of 1000 lux on the surface of the tanks. Larvae were reared in these conditions since 1 DAH until metamorphosis (benthic phase).

Tanks were divided into two groups: control and treatment, running with duplicates.

After larvae metamorphosed, they were transferred to industrial on-growing shallow square tanks with lower light conditions, 200 lux at the surface.

2.2 Feeding regime

Feeding regime of the groups was according to table 1, where control group followed a standard feeding sole regime with enriched rotifers since 3 DAH until 8 DAH, newly hatched *Artemia* nauplii (AF strain INVE, Aquaculture) from 5 - 9 DAH and enriched *Artemia* metanauplii (EG strain INVE, Aquaculture) from 8 DAH until weaning (31 DAH), with Gemma Diamond commercial feed. Treatment group feeding regime was conducted under the same conditions as the control group, with the exception of the use

of *Artemia sp.* AF nauplii (INVE, Aquaculture). Instead, rotifer feeding period was followed by direct co-feeding with *Artemia sp.* EG enriched metanauplii (INVE, Aquaculture).

Table 1 - Feeding regime for Treatment and Control groups throughout the trial (Imsland *et al.*, 2003).

DAH	Rotifers	*Artemia* AF	*Artemia* EG	Gemma Diamond
Control	3 - 8	5 - 9	8 - 31	31 - 36
Treatment	3 - 8	-	5 - 31	31 - 36

Both rotifers and *Artemia sp.* EG metanauplii were previously enriched with Larviva Multigain (Biomar, Marine Hatchery) for 20 hours, before feeding it to the larvae.

Rotifers were enriched in 100 L tanks at a density of 500 rotifers mL^{-1} at 20ºC and *Artemia* metanauplii (EG strain, Inve) were enriched in 1000 L containers with 100 metanauplii mL^{-1} at 26ºC.

Live prey was washed with UV filtered seawater before feeding it to the larvae.

Rotifers and *Artemia* were sampled twice a day to adjust prey concentration in the tanks, according to the company protocol.

Live food was distributed 4 times a day and after weaning, food distribution was increased to 8 times per day with Gemma Diamond.

2.3 Sampling

Standard length (L_{st}) and dry weight (D_W) of *Solea senegalensis* larvae were measured at 2, 5, 7, 12, 15, 20, 30 and 36 DAH. For dry weight analyses, sixty larvae were sampled from each tank. From these, twenty larvae were randomly selected to be measured using a microscope with a graduated scale. After measuring, larvae were then

rinsed in distilled water to remove any remaining salt and dried at 60ºC for 48 hours to determine dry weight. These samples were later weighted in an analytic microbalance.

To analyse fish performance condition index (K), relative growth rate (RGR) and specific growth rate (SGR) were determined.

The eye migration stage during sole metamorphosis was assessed at 2, 5, 7, 12, 15 and 20 DAH with samples of twenty larvae per tank, according to the description of Fernández-Díaz *et al.* (2001). Degrees of metamorphosis were divided according to table 2.

Table 2 - Stages of eye migration in sole metamorphosis according to Fernández-Díaz *et al.* (2001).

Stage	Description
0	Symmetrical left and right eye position
1	Asymmetrical position of the left eye and right eye, the left eye starts to migrate
2	The migrating eye reaches at maximum in the middle of the dorsal surface
3	The migrating eye can be seen from the right ocular side or migrates within the dorsal side
4	Eye translocation is completed and the orbital arch is visible

To compare both groups, eye migration index (IEM = Σ (% fish in each stage (Table 4) x stage) / 100) was calculated, according to Solbakken *et al.* (1999).

For biochemical analysis, 500 mg of larvae dry weight were sampled from each tank at the end of the experiment (36 DAH) and stored in methanol at -20ºC until further analysis in Institute of Biomedical Sciences Abel Salazar. Fatty acids results were obtained in absolute levels of FAME (lg mg^{-1} D_W) and of each individual assayed FA were transformed into relative amounts and expressed as percentages of total FAME.

For deformities analysis, thirty specimens were sampled from each tank at the end of the experiment (36 DAH). However a problem during the staining process damaged the samples and new ones were taken later on, at 79 DAH, from the on-growing production

tanks with the same trial groups (thirty individuals per tank). However, these fish had already suffered a screening at 40 DHA, in which all underdeveloped fish were removed from production. Samples were processed using a double staining method described by Gavaia *et al.* (2000), which uses Alcian Blue 8GX and Alizarin Red S, for cartilage and bone staining respectively. After staining samples were submitted to a KOH treatment in order to render soft tissues into transparency to allow observation of skeleton structures. The incidence of skeletal deformities was evaluated in each experimental group and severely affected structures (scoliosis, lordosis, kyphosis, multiple vertebral fusions or more than three anomalies per individual) were considered severe deformities.

2.4 Statistical analysis

All data presented are mean ± standard deviation (SD) of treatment and control duplicates (n=2).

To analyse the growth in both groups, relative growth rate was calculated according to the formula: $G = 100 (\ln S_2 - \ln S_1) (t_2 - t_1)^{-1}$, where S_1 and S_2 are initial and final mean length respectively in mm, and t_1 and t_2 are the days of samples (Forsythe and Van Heukelen, 1987).

Specific growth rate was also determined using the formula: SGR = 100 ((ln FBW – ln IBW) / T), where FW is the final body weight (g), IBW the initial body weight (g) and T is the duration of feeding in days (Ferguson *et al.*, 2010).

Condition factor (k) was determined by the formula: K = FBW (g) / [length (cm)] ^{3} x 100, where FBW is final body weight (Fulton, 1904).

One way ANOVA test was used to test differences between treatments, after testing for the necessary assumptions with normality Kolmogorov-Smirnof test and homogeneity variances with Levene test. Differences were considered significant when $p<0.05$. All statistical analyses were carried out using SPSS v23 software.

3 Results

3.1 Growth

All larvae used in this trial were from the same batch of the same breeding group. They were randomly distributed by all tanks and at 2 DAH had an average length of 3.28 ± 0.12 mm, and average dry weight of 0.03 ± 0.0 mg.

Feeding regimes showed significant differences ($p < 0.05$) on larval growth. Dry weight (Figure 18), at the end of the trial (36 DAH), showed significant differences between groups. Dry weight curve was analogous for both groups since the start of exogenous feeding, throughout the trial, peaking after 16 DAH. At 30 DAH, the treatment group started to display higher weight compared to the control group. This trend was maintained until the end of the trial at 36 DAH.

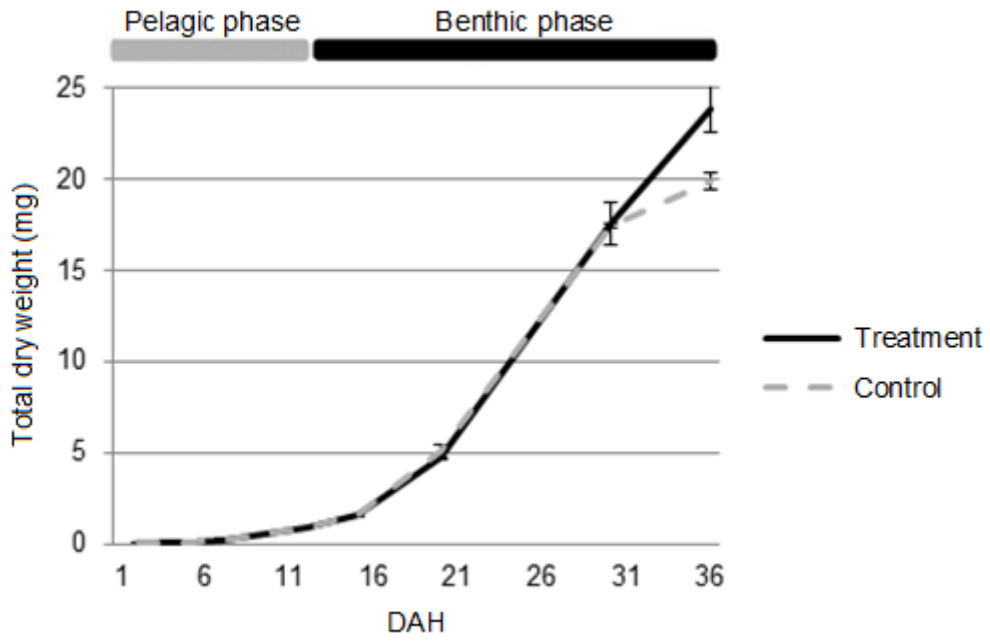

Figure 18 - Dry weight (mg) of the *Solea senegalensis* larvae throughout the trial (2 - 36 DAH). Significant differences were observed between groups at 36 DAH ($p < 0.05$). Data is expressed as mean ± SD (n = 2).

Larvae standard length (Figure 19) curve displayed the same behaviour as dry weight, with similar values throughout the trial. Despite no significant differences were recorded between groups during the 36 days of rearing, treatment group started to display a higher length after 21 DAH. This trend was maintained until the end of the trial, where the group's length uniformed once again, with no apparent differences at 36 DAH.

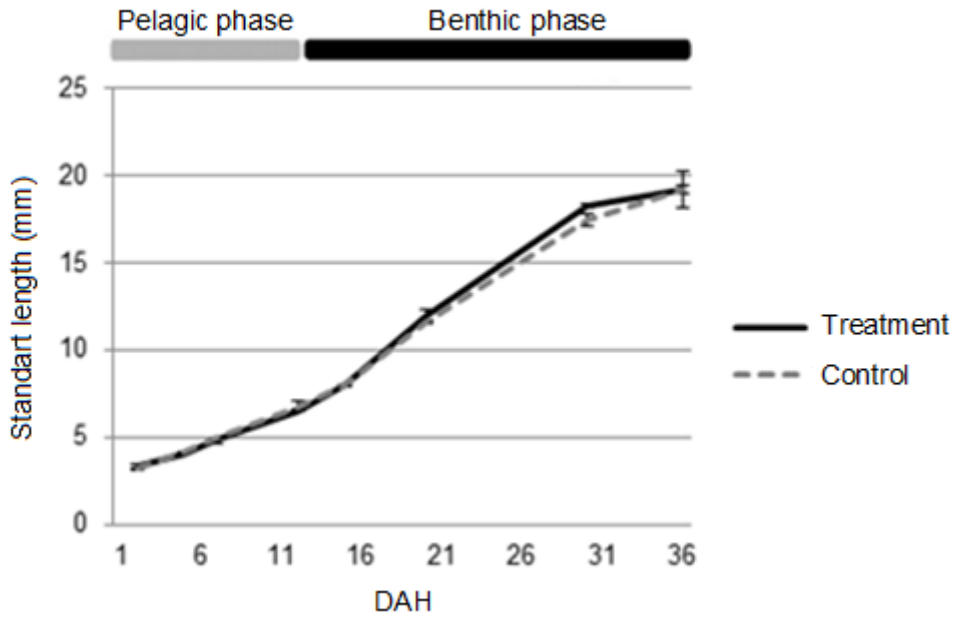

Figure 19 - Standard length (mm) of the *Solea senegalensis* larvae throughout the trial (2 - 36 DAH). No significant differences were observed in both groups ($p < 0.05$). Data is expressed as mean ± SD (n = 2).

Condition index (k) is an alternative to measure the overall health of fish by accessing their weight and length, making it a valuable tool for management decisions. Condition index showed no statistical differences ($p < 0.05$) between both groups (Table 3). Therefore, despite the bigger size of *Artemia sp.* EG strain, that could cause some constrains on fish feeding ability in the first days of exogenous feeding, larvae were not negatively affected by the alternative feeding regime.

Table 3 - Condition index for both groups between 12 DAH and the end of the trial (36 DAH). No significant differences were observed in both groups ($p < 0.05$). Data is expressed as mean ± SD (n = 2).

DAH	Control	Treatment
12	2.87 ± 0.64	3.00 ± 0.81
15	2.89 ± 0.50	3.17 ± 0.49
20	3.05 ± 0.26	2.92 ± 0.26
30	3.14 ± 0.49	2.98 ± 0.18
36	3.20 ± 0.39	3.19 ± 0.46

Dispersion calculates the group homogeneity in the tanks. Smaller values indicate good homogeneity, where larger numbers indicate more heterogeneity in the reared fish. Dispersion for both groups throughout the trial is shown in table 4. Dispersion was better at 5 DAH for control group, with the introduction of *Artemia sp.* AF in control group and *Artemia sp.* EG in treatment group, until 12 DAH. This may be explained by the difference in size of both preys (*Artemia sp.* EG and AF), though the ability of individuals of the treatment group to be able to feed on larger *Artemia sp.* EG or even rotifer size preference over *Artemia sp.* EG in the tanks during co-feeding period. However later in the trial, results reversed and showed a tendency for best homogeneity in the treatment group with some significant differences ($p < 0.05$) at 12, 20 and 36 DAH.

Table 4 - Dispersion of the *Solea senegalensis* larvae throughout the trial (2 - 36 DAH). Significant differences were observed between groups at 36 DAH. Data is expressed as mean ± SD (n = 2). Different superscript letters indicate statistical differences ($p < 0.05$) between different treatments.

DAH	Control	Treatment
2	3.55 ± 1.68	3.55 ± 1.68
5	4.71 ± 0.47	5.47 ± 1.09
7	4.00 ± 1.07	4.94 ± 0.88
12	9.09 ± 4.45^{a}	7.85 ± 2.70^{b}
15	5.24 ± 0.65^{a}	9.09 ± 0.13^{b}
20	5.67 ± 0.41^{a}	9.09 ± 0.09^{b}
30	5.69 ± 0.84	5.81 ± 1.98
36	9.68 ± 2.74^{a}	5.60 ± 0.77^{b}

Relative growth rate (% day^{-1}) and weight gain (mg / fish / day) showed no significant differences between groups, $p < 0.05$, with weight gain being slightly superior for treatment group. Specific growth rate (% day^{-1}), presented in table 5, was significantly different between treatments, with treatment group having the highest value, 19.64 ± 0.15.

Table 5 - Performance analyses of the *Solea senegalensis* larvae throughout the trial (2 - 36 DAH). Data is expressed as mean ± SD (n = 2). Different superscript letters indicate statistical differences (p < 0.05) between different treatments.

	Control	Treatment
Start of the trial		
Days post-hatching	2	2
Development stage[1]	Metamorphosis (0)	Metamorphosis (0)
D_W[2] (mg)	0.03 ± 0.0	0.03 ± 0.0
Standard length (mm)	3.28 ± 0.14	3.28 ± 0.14
End of the Trial		
Days post-hatching	36	36
Development stage[1]	Metamorphosis (4)	Metamorphosis (4)
D_W[2] (mg)	19.85 ± 0.44	23.86 ± 1.27
RGR[3] (% day^{-1})	5.20 ± 0.05	5.19 ± 0.06
Weight gain (mg / fish / day)	0.58 ± 0.01	0.70 ± 0.03
Standard length (mm)	19.19 ± 1.09	19.17 ± 0.24
SGR[4] (% day^{-1})	19.10 ± 0.07^{a}	19.64 ± 0.15^{b}

[1] Developmental stages according to Fernández-Díaz *et al.* (2001)

[2] Dry weight

[3] Relative Growth Rate

[4] Specific Growth Rate

3.2 Eye migration

Metamorphosis stage was determined according to the description of Fernández-Díaz *et al.* (2001) (Fig. 20). For statistical analysis, the eye migration index was calculated (IEM = Σ (% fish in each stage x stage) / 100), revealing that sole metamorphosis stage was not affected by the feeding regime, with no significant differences between control and treatment, $p < 0.05$.

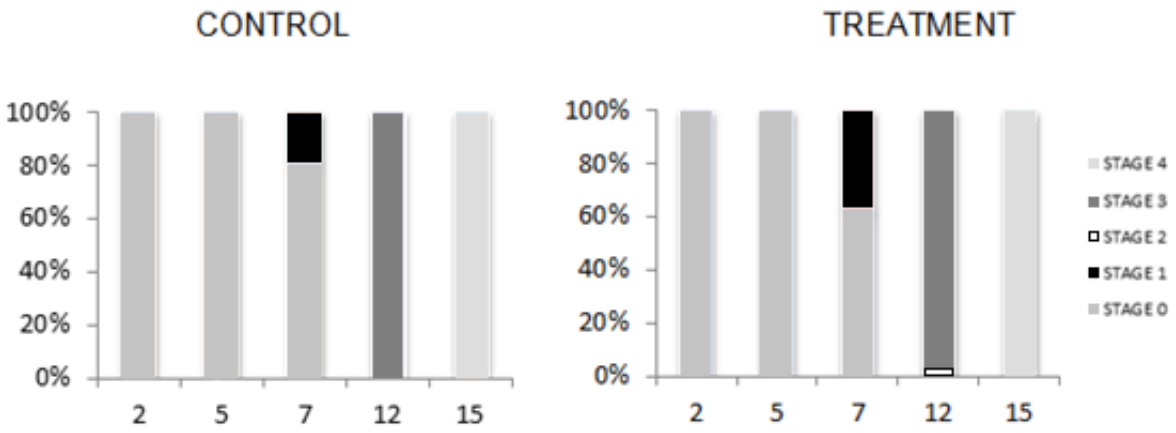

Figure 20 – Eye migration index according to Fernández-Díaz *et al.* (2001). Throughout the trial in control and treatment groups (n = 2). No significant differences were observed in both groups (p < 0.05).

3.3 Fatty acid composition

Fatty acid and lipid composition were determined for sole in the end of the trial, 36 DAH. Results are shown in Table 6 and expressed in percentage of total FAME.

Table 6 - Fatty acid composition of *Solea senegalensis* larvae at the end of the trial (36 DAH). Data is expressed as mean ± SD (n = 2). Different superscript letters indicate statistical differences (p < 0.05) between different treatments.

	Control	Treatment
% Lipids	12.29 ± 0.78	12.12 ± 0.50
Fatty acids		
12:0	0.05 ± 0.01	0.07 ± 0.01
13:0	0.01 ± 0.00[a]	0.02 ± 0.00[b]
14:0	1.44 ± 0.01	2.12 ± 0.25
15:0	0.22 ± 0.01	0.32 ± 0.04
16:0	15.44 ± 0.13	22.32 ± 2.54
17:0	0.36 ± 0.00	0.51 ± 0.05
18:0	5.60 ± 0.02	7.98 ± 0.85
20:0	0.21 ± 0.00[a]	0.31 ± 0.02[b]
21:0	0.06 ± 0.00	0.09 ± 0.01
22:0	0.33 ± 0.02	0.47 ± 0.05
00:0	0.24 ± 0.01	0.33 ± 0.03
Total saturated (Σ BCFA)	1.65 ± 0.02[a]	2.37 ± 0.22[b]
C16:1n-7	2.11 ± 0.00	3.07 ± 0.35
C17:1n-7	0.18 ± 0.00[a]	0.26 ± 0.02[b]
C18:1n-7	4.52 ± 0.01	6.56 ± 0.73
C18:1n-9	15.27 ± 0.10	22.09 ± 2.46
C20:1n-9	1.14 ± 0.01	1.68 ± 0.19
C22:1n-9	0.18 ± 0.00[a]	0.27 ± 0.02[b]
C24:1n-9	0.38 ± 0.01	0.55 ± 0.08
Total monounsaturated (Σ MUFA)	24.47 ± 0.03	35.53 ± 3.99
C16:2n-4	0.07 ± 0.01	0.09 ± 0.01
C22:1n-11	0.70 ± 0.02	1.04 ± 0.14
C18:2n-6	7.80 ± 0.14	11.33 ± 1.46
C18:3n-6	0.22 ± 0.00	0.32 ± 0.03
C20:2n-6	0.29 ± 0.00	0.39 ± 0.04
C20:3n-6	0.19 ± 0.01	0.27 ± 0.03
C20:4n-6	2.81 ± 0.05	4.05 ± 0.43
C22:5n-6	3.26 ± 0,05	4.68 ± 0.48

Total n-6 PUFA (Σ n-6)	14.55 ± 0.05	21.05 ± 2.47
C18:3n-3	11.65 ± 0.26	16.73 ± 1.66
C18:4n-3	1.43 ± 0.03[a]	2.07 ± 0.19[b]
C20:3n-3	1.02 ± 0.00	1.46 ± 0.16
C20:4n-3	0.66 ± 0.01	0.95 ± 0.10
C20:5n-3	2.41 ± 0.04	3.53 ± 0.39
C21:5n-3	0.03 ± 0.00	0.04 ± 0.00
C22:5n-3	2.56 ± 0.01	3.70 ± 0.43
C22:6n-3	11.15 ± 0.04	16.10 ± 1.61
Total n-3 PUFA (Σ n-3)	30.90 ± 0.31	44.57 ± 4.55
Total PUFA (Σ PUFA)	45.51 ± 0.27	65.71 ± 7.03
n-6 / n-3 ratio	0.47 ± 0.01	0.68 ± 0.08
DHA / EPA	4.63 ± 0.04	4.56 ± 0.76

3.4 Sole Quality

Sole quality was accessed with fish from the trial at 79 DAH. Percentage of affected fish and fish with severe deformities were higher for control group (Table 7), however there were no statistical differences between groups ($p < 0.05$).

Table 7 - Percentage of affected fish and fish with severe deformities in both groups (n = 2). No significant differences were observed in both groups ($p < 0.05$).

	Afected fish	**Fish affected with severe deformities**
Treatment	38.3%	3.3%
Control	53.3%	10%

Most affected body regions in each group (Figure 21) were the caudal fin structure for both dietary treatments, followed by the haemal vertebrae for the control group and the caudal vertebrae for the treatment group.

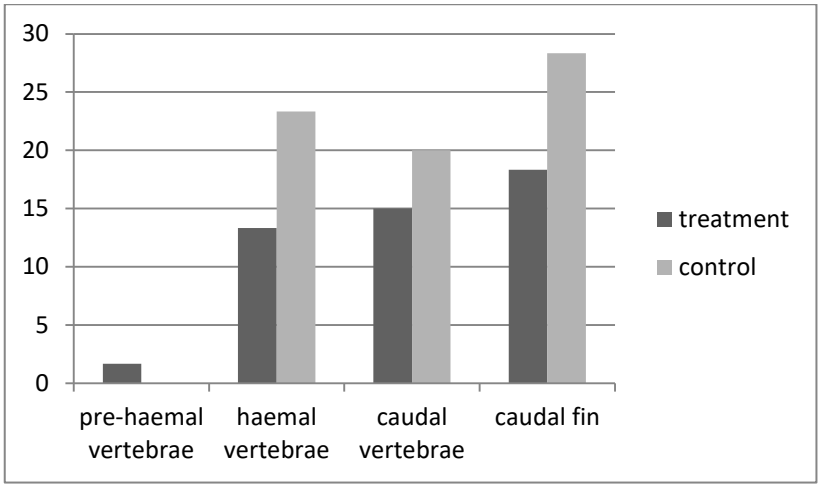

Figure 21 - Affected body regions for both groups (%). No significant differences were observed in both groups ($p < 0.05$).

Analysed fish were distributed according to the number of deformities (Figure 22). Control group displayed a higher occurrence of individuals affected with one, two, three and four deformities.

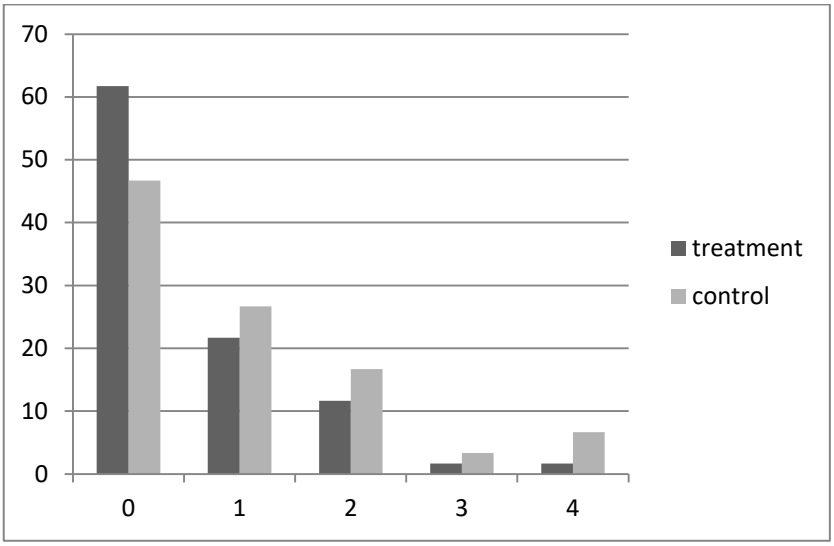

Figure 22 - Distribution of analysed fish according to the number of deformities observed in each individual (%).

Malformations were divided into nine categories according to the place of incidence: trunk vertebrae, caudal vertebrae, arches, neural spines, haemal spines, parapophysis, caudal fin rays, hypurals and epurals. Figure 23 shows the distribution of the deformities in the affected structures.

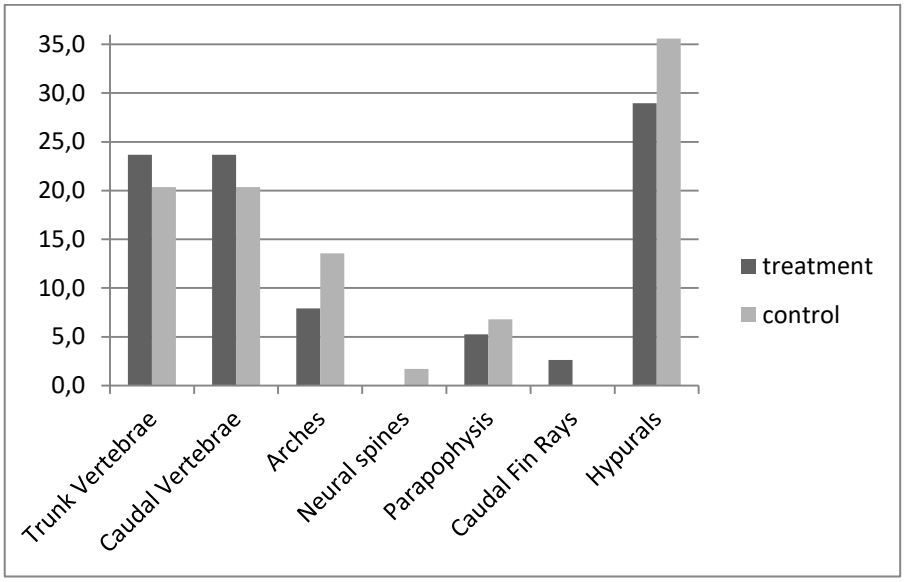

Figure 23 – Distribution of the abnormalities detected according to the affected structures, with high incidence in the caudal structures (%).

4. Discussion

At the beginning of exogenous feeding, larvae switch their source of nutrients and energy required to continue their development from yolk reserves to ingested food (Yúfera and Darias, 2007). At this point mouth and anus are open and yolk reserves are almost depleted (5%). Starvation in this critical period can lead to the point of no return, with massive mortalities. This point mainly depends on temperature (McGurk, 1984; Arul, 1991; Dou *et al.*, 2002; Dou *et al.*, 2005) and larval length (Miller *et al.*, 1988) and it's referred to a moment of irreversible starvation, where 50% of the larvae can no longer start feeding (Gwak and Tanaka, 2001; Dou *et al.*, 2002). At this phase larvae digestive system differentiation is not completed and consists in a simple tube. Liver and pancreas are also functional and digestion occurs in an alkaline environment with the help of pancreatic enzymes. Larvae are able to ingest, digest and assimilate ingested food, being only limited by their mouth gape, swimming ability and hunting success. Thus the onset of feeding is a crucial moment in larvae development correlated with massive mortalities. Conditions of starvation, unsuitable food quality or feeding procedures can drastically reduce survival in the first days or weeks of rearing. Survival is variable between fish species, under optimal conditions up to 85% is registered for *Solea senegalensis* (Yúfera *et al.*, 2005) during the first weeks after first feeding.

At the start of exogenous feeding, marine fish larvae are mainly visual predators with their eyes being pigmented but only equipped with single cones, the rod cells will appear later in the development (Kvenseth *et al.*, 1996; Pankhurst and Eagar, 1996; Helvik *et al.*, 2001; Shand *et al.*, 2002; Kawamura *et al.*, 2003; Hall *et al.*, 2004). Larvae also have some neuromasts to detect movement in the water and olfactory cells for chemical stimuli to help predation (Yin and Blaxter, 1987; Boglione *et al.*, 2003). Taste buds appear later in the development, though larvae select prey mainly by its size than taste or other characteristics. Smaller preys are usually preferred with 25 - 50% of the mouth gape being the most appropriate (Shirota, 1970; Fernández-Díaz *et al.*, 1994; Busch, 1996; Munk, 1997; Cunha and Planas, 1999; Østergaard *et al.*, 2005). The mouth gape increases substantially in the first days of rearing (Shirota, 1970; Fernández-Díaz *et al.*, 1994; Doi *et al.*, 1997), allowing larvae to capture increasing size of prey (Polo *et al.*, 1992; Olsen *et al.*, 2000).

Sole larvae are equipped with a wider mouth compared to other species that live in temperate habitats, like Gilthead seabream. This anatomical feature allows sole larvae to ingest larger prey, therefore securing a higher energy per consumed prey and allowing a

better balance during early development between energy used to capture and ingest the prey, when compared to Gilthead seabream, with both species growing continuously in the presence of prey (Parra and Yúfera, 2001). Larger prey allows *Solea senegalensis* to achieve higher metabolic efficiency, without increasing ingestion rate, translating in faster development and higher predatory abilities like increased sustained and burst swimming speeds and better visual efficiency (Keast and Webb, 1966; Webb, 1976; Beamish, 1978; Blaxter, 1986). This is one of the main reasons of the fast larval growth of *Solea senegalensis* in comparison with Gilthead seabream (Parra and Yúfera, 2001).

The nutritional effectiveness of a food organism is primarily determined by its ingestibility, as their size and configuration (Léger *et al.*, 1986). If opportunity is given Lemon sole larvae (*Microstomus Kitt*) will prefer smaller preys, as trochopores over rotifers and rotifers over *Artemia* nauplii (Howell, 1972). According to this, *Solea senegalensis* larvae protocols include the feeding with rotifers during the first days of rearing, followed by the use of small size *Artemia* of newly hatched non-enriched nauplii due to larvae small size. The escape response of prey is also related to its body size, with reaction distance increasing alongside with swimming performance (Folkvord and Hunter, 1986; Blaxter and Fuiman, 1990). As a result the size of prey consumed generally increases with increasing predator size (Keast and Webb, 1966; Popova, 1967; Popova, 1978; Nielsen, 1980; Persson, 1990; Juanes *et al.*, 1994), due to increasing predating abilities. However some studies show that sometimes predators can select smaller prey when given the choice (Gillen *et al.*, 1981; Hoyle and Keast, 1987; Hart and Hamrin, 1990; Juanes *et al.*, 1994), probably due to less hunting efforts.

The early substitution of *Artemia sp.* AF nauplii instar I for a larger *Artemia sp.* EG enriched instar II could not only limit the capability of sole larvae to prey on this *Artemia* due to its larger size, causing a possible selection of the bigger specimens in the first days of rearing, but also causing a prey preference of rotifers over larger *Artemia sp.* EG during co-feeding, with a resulting lower energy uptake for prey item consumed.

Howell (1973) reported that larvae fed with exclusively rotifers and rotifers/*Artemia* combination, did not show any positive effects in survival, due to the presence of smaller prey (Imsland *et al.*, 2003). Accordingly the results show that the treatment group was not negatively affected by dietary treatment, even though prey length and body volume disparity between *Artemia sp.* AF (480 µm) and *Artemia sp.* EG (500 - 600 µm) was reasonable.

In the present work different feeding regimens affected larvae growth. At the end of the trial both groups showed no significant differences ($p < 0.05$) in standard length, but displayed significant differences ($p < 0.05$) in dry weight, higher in treatment group. At 31 DAH, the treatment group started to display higher dry weight, a trend that maintained until the end of the trial (36 DAH), with treatment group having 23.86 ± 1.27 mg and control group 19.85 ± 0.44 mg. Standard length was similar between groups, since 2 DAH until 21 DAH, where the treatment group had slightly higher length, compared to the control group, until 31 DAH. At the end of the trial (36 DAH) both groups presented similar lengths.

Stage of metamorphosis was accessed at 2, 5, 7, 12 and 15 DAH according to the methodology of Fernández-Díaz *et al.* (2001). Eye migration index showed no significant differences between treatments. Metamorphosis stage was uniform for both groups throughout the trial, with the exception of 7 DAH, where the treatment group had a higher proportion of larvae already into stage 1, approximately 40%, compared to the control group, with 20% of total larvae into stage 1.

Dispersion was significantly different between groups at 12 DAH, with control group having the higher dispersion (control 9.09 ± 0.45 and treatment 7.85 ± 2.7), probably due to later introduction of *Artemia sp.* EG in the feeding schedule (8 DAH). Sole larvae from the treatment group may have benefited from their prior learning experience with prey by continuously feeding on *Artemia sp.* EG and not had to switch from AF to EG *Artemia sp.*, thus not affecting larval performance (Cox and Pankhurst, 2000). By the end of the trial (36 DAH), treatment group displayed a significantly different lower dispersion population in the tanks. *Artemia sp.* EG size did not appear to cause any negative effects on larvae early development. Even though fish final length was not affected by dietary treatments, larval dry weight was significantly affected and it had a positive effect in reducing population dispersion, as the treatment group reflected a more homogenous population in the tanks by the end of the trial (36DAH), (5.60 ± 0.77) compared to the control group (9.68 ± 2.74). A desirable feature for fish culture, that can reduce the number of necessary size grading during production, thus reducing animal handling that can induce stress in the fish, therefore increasing fish performance.

Condition index is used to estimate fish overall health, and should always be superior to 1. Throughout the trial condition index presented no significant differences ($p < 0.05$) between groups. At 15 DAH the condition index was higher for the treatment group (3.17 ± 0.49) compared to the control group (2.89 ± 0.50). At 30 DAH condition was reversed and control group displayed superior index (3.14 ± 0.49) than treatment group

(2.98 ± 0.18). At the end of the trial the index values were identical with no significantly differences.

Relative growth rate (% day^{-1}) and weight gain (mg / fish / day) weren't affected by dietary treatments and showed no significant differences ($p < 0.05$) between groups. However specific growth rate (% day^{-1}) was significantly different ($p < 0.05$) higher for treatment group at the end of the trial (36 DAH).

Artemia size appeared not to be a bottleneck for smaller *Artemia sp.* AF instar I substitution by larger enriched *Artemia sp.* EG instar II, whose nutritional quality can be manipulated through the enrichment process. However *Artemia* are not passive fatty acid carriers, due to their own physiological needs. These can affect the original enrichment composition, by retro converting DHA into EPA and by redistributing the fatty acids among lipid classes with high unpredictability (Navarro *et al.*, 1999). Boglino *et al.* (2012) noticed that diets with high and medium amounts of DHA contained low levels of OA and MUFA (Villalta *et al.*, 2005a), which are commonly used as energy for larval growth and development, due to the fact that SFA and MUFA can be easily catabolized in fish, compared to DHA, which is not easily catabolized via β-oxidation (Sargent *et al.*, 2002). Furthermore, diets with higher EPA levels led to the accumulation of EPA and DPA in the tissues, probably due to sole being unable to elongate and desaturate them into DPA at a significant rate (Morais *et al.*, 2004).

Insufficient supply of essential fatty acids for fish larvae can lead to constraint growth, pathologies and mortalities (Sargent *et al.*, 1995). For instance, DHA is the most abundant FA in cerebral tissue and in the retina (Mourente, 2003), as a result dietary deficiencies can induce permanent impaired visual performance and reduced feeding (Bell *et al.*, 1995), and lead to abnormal schooling behaviour in fish larvae (Hossain *et al.*, 2002). However *Solea senegalensis* larvae appear to have low requirements for dietary DHA. As several studies have demonstrated the ability of *Solea senegalensis* larvae to survive, grow and metamorphose on *Artemia* with low content of DHA, but containing other n-3 PUFA (Morais *et al.*, 2004; Villalta *et al.*, 2005b), namely EPA as primary energy source of n-3 PUFA (Villalta *et al.*, 2005a). This may be explained by sole natural preys in their habitat, as benthic fauna is rich in EPA. Similar results have been reported with Japanese flounder (Izquierdo *et al.*, 1992), common sole (Tzoumas, 1988) and plaice (Dickey-Collas and Geffen, 1992). Other flatfish species with longer larvae periods like turbot (Le Milinaire, 1984) and yellowtail flounder (Copeman *et al.*, 2002), require exogenous supply of DHA and EPA for normal growth and survival. Probably the low requirements of dietary DHA explain its success in rearing *Solea senegalensis* with

different types of *Artemia*, some with deficient amounts of EFA (Sargent *et al.*, 1999). However *Solea senegalensis* may have different fatty acid needs according to its life stage, pre-metamorphosis or post-metamorphosis. During pre-metamorphic stage, pelagic larvae have access to large amounts of DHA sources in the pelagic food chain (Kainz *et al.*, 2004). Sole larvae feed mainly on copepods (80.3% stomach content) and veliger bivalve (13.4% stomach content) (Holland, 1978; Sargent and Falk-Petersen, 1988; Morehead *et al.*, 2005). These organisms are rich in n-3 PUFA, mainly DHA. After metamorphosis, around 1000 µg D_W energy used for metabolism is increased and the energy used for growing diminishes (Parra, 1998), as sole changes its ecologic niche and feeding habits by migrating to the benthic zone, where it has an abundance of diatom algae, rich in EPA and 16:0 fatty acid (Kates and Volcani, 1966; Graeve *et al.*, 1997), and polychaete (75% stomach content), also rich in EPA (Cabral, 2000), which is probably used as a main source of n-3 PUFA (Villalta *et al.*, 2005a). Hence although some results seem to fail to demonstrate clear effects of PUFA in the development and survival of fish larvae (Morais *et al.*, 2004), these could only become apparent in later stages of life (Howell *et al.*, 1995). Villata et al. (2005) compared the effects of gradual concentration of dietary DHA in *Solea senegalensis,* and higher DHA content diets resulted in an accumulation of fewer lipids in the tissue, namely Oleic acid (OA) (18:1). These may be responsible for the lower growth in the fish. DPA is the product of the quick EPA elongation in fish, further elongation, desaturation and chain shortening results in DHA, however this is a more complicated process and probably occurs in non-significant rates in fish for the production of DHA (Sargent *et al.*, 1995). Accumulation of DPA was reported in studies with insufficient dietary levels of DHA (Izquierdo *et al.*, 1992; Bell *et al.*, 1995; Bransden *et al.*, 2004).

Fatty acid profile of the fish at the end of the trial appeared highest in the enriched *Artemia sp.* EG treatment group, whereas the control group had overall the lowest values. Larvae fed with enriched *Artemia sp.* EG (treatment group) had higher levels of EPA (20:5n-3) and DPA (22:5n-3). A surplus of EPA in the tissues can have a negative effect on larval development, so EPA levels should be in the range of 3 - 4 of TFA, to enhance larval growth and survival (Léger *et al.*, 1986; Izquierdo *et al.*, 2000). The EPA levels for treatment group were between this range, 3.53 ± 0.39, however EPA levels for control group were below the 3 - 4 of TFA range. DHA levels didn't reflect significant differences but were higher for treatment group. The percentage of lipid composition in fish was not affected by dietary treatments.

Some of the fish pigmentation disorders causes have been identified as broodstock management, husbandry and physiological modifications caused by environmental conditions like photoperiod, light intensity, temperature, substrate and colour of the tanks during incubation and larval rearing (Lebegue, 1982). Dietary ARA is important in the control of pigmentation in larvae (Villalta *et al.*, 2005a) and high levels of this fatty acid can lead to hypopigmentation (Lund *et al.*, 2008). In the end of the trial no fish presented pigmentation disorders. ARA levels weren't significantly different between dietary treatments, but it was higher for the treatment group compared to the control, 4.05 ± 0.43 and 2.81 ± 0.05 of total FAME respectively.

Regarding sole quality, the samples of the fish skeletons were stained using the Alizarin Red-Alcian Blue double staining method, to look for any deformities. This technique is also used to visualize bone and cartilage during developments in sea bass (Boglione *et al.*, 1993; Marino *et al.*, 1993) and gilthead sea bream (Faustino and Power, 1998; Gavaia *et al.*, 2000).

At 12 - 13 DAH starts the development of caudal complex and vertebral column, alongside with the urostyle torsion and the migration of the left eye. In the Japanese flounder, the flexion of the notochord is also related to the development of the hypuralia (Hosoya and Kawamura, 1998), which is perhaps explained by the importance of these structures for larvae feeding and swimming abilities. In *Solea senegalensis* the caudal fin is the first structure to develop, acquiring full meristic count after 6.1 mm (standard length), followed by anal, dorsal and then paired fins. This sequence of development was also reported in red sea bream (Kohno *et al.*, 1983), milkfish (Taki *et al.*, 1987), common dentex (Koumoundouros *et al.*, 1999) and in the Japanese flounder (Hosoya and Kawamura, 1998).

Overall incidence of deformities was not significantly affected by dietary treatments, although control group had more affected fish (53.3%) than treatment group (38.3%). These results were similar to those reported by Gavaia *et al.* (2002), with an incidence of 44% hatchery-reared *Solea senegalensis*. Control group also had the most affected fish with severe deformities.

The most affected structures in both groups were trunk vertebrae, caudal vertebrae and the caudal region, with fusion of the hypurals. Control group displayed higher deformities in the caudal vertebra (fusion) and treatment group had a higher number of fish with fusion of the hypurals in the caudal structure. Gavaia *et al.* (2002) also reported a high incidence of vertebral fusions in *Solea senegalensis*, suggesting that the

development of these structures could be the most susceptible to rearing conditions in captivity. Furthermore these deformities can be easily visible in the fish, as they modify the shape and length of the fish, a serious concern for market. We can hypothesize that fish from the control group display a higher risk of developing macroscopic alterations to their body form later on in their development, due to their higher incidence of vertebral fusions, and have to be discarded from production due to market demands. Examined fish didn't show any deformities that severely modify their shape. This may be explained by the fact that the fish used for deformities assessment were previously subjected to a size grading, therefore shape or size severely affected fish could have been already discarded from production. We can then assume that the deformities identified in our results, are the ones that stay in the production at least past 79 DAH and move on unidentified to the pre-fattening phase of Safiestela production, increasing possible economic losses.

IV. Conclusion

Aquacria Piscicolas, S.A. previously produced turbot (*Psetta maxima),* however juveniles were bought from other hatcheries in Spain. With the construction of Acuinova, S.A. (Pescanova) facilities in Portugal, the company shifted their strategy and undergone facilities requalification's in order to produce *Solea senegalensis*. As a result Safiestela, S.A. was created and started their production of *Solea senegalensis* juveniles in 2012, after also undergoing severe requalification to their facilities, that were previously acquired from another company.

The internship at Safiestela was an amazing experience both in professional and personal development, where we could experience the difficulties and challenges that each section of the production posed to both the company and the technicians. It allowed learning hands-on, by working in the different stages of production since broodstock, larvae, live feed production, juveniles, production systems and even the maintenance of recirculation equipment in these productions. It was an important learning experience that will dictate our future in this industry.

The trial concerning the substitution of *Artemia sp.* AF nauplii for *Artemia sp.* EG metanauplii in *Solea senegalensis* was a success, with larvae growth being significantly affected by dietary treatments. Larvae from the treatment group displayed a higher dry weight, better homogeneity and reduced incidence of deformities at the end of the trial (36 DAH). The fatty acid profile results were also encouraging as larvae from the treatment group also displayed overall higher values of total FAME percentage.

Production costs are heavily affected by the rearing of the larval stage, mainly due to the live feed costs. Although *Artemia sp.* AF (Inve, Aquaculture) presents itself as great food item for the first days of feeding, due to its high nutritional value and small size, it also has a high market price, compared to other commonly used strains, as EG strain (Inve, Aquacuture). This trial showed that sole larvae could start feeding on enriched rotifers, followed by direct co-feeding with EG enriched metanauplii until weaning is possible, without the use of an intermediary size prey. As a result Safiestela can alter their feeding protocols, which include the use of the expensive AF strain that brings no apparent benefits to the larval rearing of sole, though resulting in positive decreasing of production costs concerning *Solea senegalensis* production. The use of a single *Artemia* strain also reduces labour in the live feed production area, optimizing the time and tasks in this section, therefore reducing labour costs for the company.

To fully understand the impact of this dietary treatment, longer trials are needed to carefully follow the development of the fish. In the future more knowledge about specific

species larvae requirements need to be understood to fully optimize larval rearing, including live feeds enrichment products for species specific during early development.

In the last years a lot of research has been directed into the development of intensive copepod production indoor systems, and in a near future, they could change the industry, with the commercial production of dormant copepods eggs.

Eventually the optimization of formulated feeds will replace live feeds in aquaculture productions, though removing high costs of cultures and the intense labour necessary to keep them. However until them, we can estimate that *Artemia* and rotifers will still be used for several years, so optimization of enrichment products and feeding protocols will be a necessity.

References

Abellan, E. & Basurco, B. (1999). Marine finfish species diversification: current situation and prospects in Mediterranean aquaculture. CIHEAM-IAMZ.

Agulleiro, M. (2007). Fisiología de la reproducción del lenguado Senegalés (*Solea senegalensis*): mecanismos endocrinos y aplicaciones en acuicultura. *PhD Thesis, Facultad de Ciencias Biológicas, Universidad de Valencia, Spain.*

Ambrosio, P. P., Costa, C., Sánchez, P. & Flos, R. (2008). Stocking density and its influence on shape of Senegalese sole adults. *Aquaculture International* 16(4): 333-343.

Andrade, J. P. d. A. (1990). A importância da Ria Formosa no ciclo biológico de *Solea senegalensis* (Kaup, 1858), *Solea vulgaris* (Quencel, 1806), *Solea lascaris* (Risso, 1868) e *Microchirus azevia* (Capello, 1868). *PhD Thesis, University of Algarve, Portugal.*

Anguis, V. & Canavate, J. P. (2005). Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture* 243(1): 133-145.

Arias, A. M. & Drake, P. (1990). Estados juveniles de la ictiofauna en los caños de las salinas de la Bahía de Cádiz. *Instituto de Ciencias Marinas de Andalucía (ICMAN).*

Arjona, F. J., Ruiz-Jarabo, I., Vargas-Chacoff, L., del Río, M. P. M., Flik, G., Mancera, J. M. & Klaren, P. H. (2010). Acclimation of *Solea senegalensis* to different ambient temperatures: implications for thyroidal status and osmoregulation. *Marine Biology* 157(6): 1325-1335.

Arul, V. (1991). Effects of delayed feeding on growth and survival of *Channa striatus* (Bloch) larvae. *Aquaculture Research* 22(4): 423-434.

Baynes, S., Howell, B., Beard, T. & Hallam, J. (1994). A description of spawning behaviour of captive Dover sole, *Solea solea* (L.). *Netherlands Journal of Sea Research* 32(3-4): 271-275.

Beamish, F. (1978). Swimming capacity. In: Hoar WS, Randall DJ (eds) Fish physiology. Academic Press, New York, p 101–187.

Bedoui, R. (1997). *Solea senegalensis* (Kaup, 1858). elevage, comportement trophique et morpho-histologie. *These du neme cycle* 129.

Bell, J., McEvoy, L., Estevez, A., Shields, R. & Sargent, J. (2003). Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture* 227(1): 211-220.

Bell, M. V., Batty, R. S., Dick, J. R., Fretwell, K., Navarro, J. C. & Sargent, J. R. (1995). Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (*Clupea harengus* L.). *Lipids* 30(5): 443-449.

Bengtson, D. A. (2003). Status of marine aquaculture in relation to live prey: past, present and future. *Live feeds in marine aquaculture*: 1-16.

Bertotto, D., Barbaro, J., Francescon, A., Richard, J., Libertini, A. & Barbaro, A. (2006). Induced spawning in common sole (*Solea solea* L.). *Aquaculture Research* 37(4): 423-427.

Blanco-Vives, B., Vera, L. M., Ramos, J., Bayarri, M. J., Mananos, E. & Sánchez-Vázquez, F. J. (2011). Exposure of larvae to daily thermocycles affects gonad development, sex ratio, and sexual steroids in *Solea senegalensis*, Kaup. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 315(3): 162-169.

Blaxter, J. (1986). Development of sense organs and behavior of teleost larvae with special reference to feeding and predator avoidance. *Transactions of the American Fish Society* 115:98–114.

Blaxter, J. & Fuiman, L. (1990). The role of the sensory systems of herring larvae in evading predatory fishes. *Journal of the Marine Biological Association of the United Kingdom* 70(02): 413-427.

Boglino, A., Darias, M. J., Ortiz-Delgado, J. B., Özcan, F., Estévez, A., Andree, K. B., Hontoria, F., Sarasquete, C. & Gisbert, E. (2012). Commercial products for Artemia enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture* 324: 290-302.

Boglione, C., Gagliardi, F., Scardi, M. & Cataudella, S. (2001). Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). *Aquaculture* 192(1): 1-22.

Boglione, C., Gavaia, P., Koumoundouros, G., Gisbert, E., Moren, M., Fontagné, S. & Witten, P. E. (2013a). Skeletal anomalies in reared European fish larvae and juveniles. Part 1: normal and anomalous skeletogenic processes. *Reviews in Aquaculture* 5(1): 99-120.

Boglione, C., Giganti, M., Selmo, C. & Cataudella, S. (2003). Morphoecology in larval fin-fish: a new candidate species for aquaculture, *Diplodus puntazzo* (Sparidae). *Aquaculture International* 11(1-2): 17-41.

Boglione, C., Gisbert, E., Gavaia, P., E Witten, P., Moren, M., Fontagné, S. & Koumoundouros, G. (2013b). Skeletal anomalies in reared European fish larvae and juveniles. Part 2: main typologies, occurrences and causative factors. *Reviews in Aquaculture* 5(1): 121-167.

Boglione, C., Marino, G., Bertolini, B., Rossi, A., Ferreri, F. & Cataudella, S. (1993). Larval and postlarval monitoring in sea bass: morphological approach to evaluate finfish seed quality. *EAS Special Publication*.

Bolker, J. & Hill, C. (2000). Pigmentation development in hatchery-reared flatfishes. *Journal of Fish Biology* 56(5): 1029-1052.

Borges, P., Oliveira, B., Casal, S., Dias, J., Conceiçao, L. & Valente, L. M. (2009). Dietary lipid level affects growth performance and nutrient utilisation of Senegalese sole (*Solea senegalensis*) juveniles. *British Journal of Nutrition* 102(07): 1007-1014.

Bransden, M., Dunstan, G., Battaglene, S., Cobcroft, J., Morehead, D., Kolkovski, S. & Nichols, P. (2004). Influences of dietary n− 3 long-chain PUFA on body concentrations of 20: 5n− 3, 22: 5n− 3, and 22: 6n− 3 in the larvae of a marine teleost fish from Australian waters, the striped trumpeter (*Latris lineata*). *Lipids* 39(3): 215-222.

Bromage, N. R. & Roberts, R. J. (1995). *Broodstock management and egg and larval quality. Blackwell Science Ltd.*

Busch, A. (1996). Transition from endogenous to exogenous nutrition: larval size parameters determining the start of external feeding and size of prey ingested by Ruegen spring herring *Clupea harengus*. *Oceanographic Literature Review* 8(43): 828.

Cabral, H. (2000). Comparative feeding ecology of sympatric *Solea solea* and *S. senegalensis*, within the nursery areas of the Tagus estuary, Portugal. *Journal of Fish Biology* 57(6): 1550-1562.

Cahu, C. & Infante, J. Z. (2001). Substitution of live food by formulated diets in marine fish larvae. *Aquaculture* 200(1): 161-180.

Cahu, C., Infante, J. Z. & Takeuchi, T. (2003). Nutritional components affecting skeletal development in fish larvae. *Aquaculture* 227(1): 245-258.

Cañavate, J. P. (2005).Opciones del lenguado senegalés (*Solea senegalensis*) para diversificar la acuicultura marina. In *Proceeding of X Congreso Nacional de Acuicultura. Universidad Politécnica de Valencia, Spain*, 340-347.

Canavate, J. P. & Fernández-Dıaz, C. (1999). Influence of co-feeding larvae with live and inert diets on weaning the sole *Solea senegalensis* onto commercial dry feeds. *Aquaculture* 174(3): 255-263.

Cañavate, J. P., Zerolo, R. & Fernández-Díaz, C. (2006). Feeding and development of Senegal sole (*Solea senegalensis*) larvae reared in different photoperiods. *Aquaculture* 258(1): 368-377.

Cardeira, J., Bensimon-Brito, A., Pousão-Ferreira, P., Cancela, M. & Gavaia, P. J. (2012). Lordotic-kyphotic vertebrae develop ectopic cartilage-like tissue in Senegalese sole (*Solea senegalensis*). *Journal of Applied Ichthyology* 28(3): 460-463.

Chen, W. M. & Purser, G. (2001). The effect of feeding regime on growth, locomotor activity pattern and the development of food anticipatory activity in greenback flounder. *Journal of Fish Biology* 58(1): 177-187.

Clement, P., Wurdak, E. & Amsellem, J. (1983).Behavior and ultrastructure of sensory organs in rotifers. In *Biology of Rotifers*, 89-129: *Springer*.

Conceição, L. E., Ribeiro, L., Engrola, S., Aragão, C., Morais, S., Lacuisse, M., Soares, F. & Dinis, M. T. (2007). Nutritional physiology during development of Senegalese sole (*Solea senegalensis*). *Aquaculture* 268(1): 64-81.

Conceição, L. E., Yúfera, M., Makridis, P., Morais, S. & Dinis, M. T. (2010). Live feeds for early stages of fish rearing. *Aquaculture Research* 41(5): 613-640.

Copeman, L., Parrish, C., Brown, J. & Harel, M. (2002). Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid

composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. *Aquaculture* 210(1): 285-304.

Costas, B., Conceição, L. E., Dias, J., Novoa, B., Figueras, A. & Afonso, A. (2011). Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). *Fish Shellfish Immunol* 31(6): 838-847.

Cox, E. & Pankhurst, P. (2000). Feeding behaviour of greenback flounder larvae, *Rhombosolea tapirina* (Günther) with differing exposure histories to live prey. *Aquaculture* 183(3): 285-297.

Cunha, I. & Planas, M. (1999). Optimal prey size for early turbot larvae (*Scophthalmus maximus* L.) based on mouth and ingested prey size. *Aquaculture* 175(1): 103-110.

Day, O., Howell, B. & Jones, D. (1997). The effect of dietary hydrolysed fish protein concentrate on the survival and growth of juvenile Dover sole, *Solea solea* (L.), during and after weaning. *Aquaculture Research* 28(12): 911-921.

Dhert, P. & Sorgeloos, P. (1995). Live feeds in aquaculture. *Infofish International* 2: 31Á39.

Dickey-Collas, M. & Geffen, A. (1992). Importance of the fatty acids 20: 5ω 3 and 22: 6ω 3 in the diet of plaice (*Pleuronectes platessa*) larvae. *Marine Biology* 113(3): 463-468.

Dinis, M. (1992). Aspects of the potential of *Solea senegalensis* Kaup for aquaculture: larval rearing and weaning to an artificial diet. *Aquaculture Research* 23(4): 515-520.

Dinis, M., Metallier, R. & Rodeia, J. (1987).Weaning of *Solea senegalensis* Kaup 1858 with rehydratable pellets. In *Nato Workshop on Nutrition, INIP Publ*, Vol. 12, 139-147.

Dinis, M., Ribeiro, L., Conceiçao, L. & Aragao, C. (2000). Larvae digestion and new weaning experiments in *Solea senegalensis*. *Cahiers Options Méditerranéenes* 47: 193-204.

Dinis, M. T. (1986). Quatre Soleidae de l'Estuaire du Tage. Reproduction et Croissance. Essai d'elevage de *Solea senegalensis* (kaup, 1858). *PhD thesis, Université de Bretagne Occidentale, France.*

Dinis, M. T. & Reis, J. (1995). Culture of *Solea spp.* In: Workshop on Diversification in aquaculture, Cyprus, 14-17 June. *Cahiers Options Méditerranéennes* 16, 1-7.

Dinis, M. T., Ribeiro, L., Soares, F. & Sarasquete, C. (1999). A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. *Aquaculture* 176(1): 27-38.

Dinis, M. T., Soares, F., Conceição, L., Cañavate, J. P., Anguis, V., Ferreira, P. P., Engrola, S., Navas, J. I., Fernández, J. L. & Hachero, I. (2007). *Manual de cultivo de lenguado y otros peces planos. Junta Andalucia.*

Divanach, P., Boglione, C., Menu, B., Koumoundouros, G., Kentouri, M. & Cataudella, S. (1996). Abnormalities in finfish mariculture: an overview of the problem, causes and solutions. *Seabass and Seabream Culture: Problems and Prospects*: 45-66.

Doi, M., Ohno, A., Kohno, H., Taki, Y. & Singhagraiwan, T. (1997). Development of feeding ability in Red snapper *Lutjanus argentimaculatus* early larvae. *Fisheries science* 63(6): 845-853.

Dou, S., Masuda, R., Tanaka, M. & Tsukamoto, K. (2002). Feeding resumption, morphological changes and mortality during starvation in Japanese flounder larvae. *Journal of Fish Biology* 60(6): 1363-1380.

Dou, S., Masuda, R., Tanaka, M. & Tsukamoto, K. (2005). Effects of temperature and delayed initial feeding on the survival and growth of Japanese flounder larvae. *Journal of Fish Biology* 66(2): 362-377.

Drake, P., Arias, A. M. & Rodríguez, R. B. (1984). Cultivo extensivo de peces marinos en los esteros de las salinas de San Fernando (Cádiz).: *II. Características de la producción de peces.* CENIP.

Drewes, C. (2005).Quantitative investigations of hatching in brine shrimp cysts. *Iowa states University, Ames.*

Engrola, S., Conceição, L. E., Dias, L., Pereira, R., Ribeiro, L. & Dinis, M. T. (2007). Improving weaning strategies for Senegalese sole: effects of body weight and digestive capacity. *Aquaculture Research* 38(7): 696-707.

Engrola, S., Figueira, L., Conceição, L. E., Gavaia, P. J., Ribeiro, L. & Dinis, M. T. (2009a). Co-feeding in Senegalese sole larvae with inert diet from mouth opening promotes growth at weaning. *Aquaculture* 288(3): 264-272.

Engrola, S., Mai, M., Dinis, M. T. & Conceição, L. E. (2009b). Co-feeding of inert diet from mouth opening does not impair protein utilization by Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture* 287(1): 185-190.

Estevez, A. & Kanazawa, A. (1995). Effect of (n-3) PUFA and vitamin A Artemia enrichment on pigmentation success of turbot, *Scophthalmus maximus* (L). *Aquaculture Nutrition* 1(3): 159-168.

Estevez, A., McEvoy, L., Bell, J. & Sargent, J. (1998). Effects of temperature and starvation time on the pattern and rate of loss of essential fatty acids in Artemia nauplii previously enriched using arachidonic acid and eicosapentaenoic acid-rich emulsions. *Aquaculture* 165(3): 295-311.

Estevez, A., McEvoy, L. A., Bell, J. G. & Sargent, J. R. (1999). Growth, survival, lipid composition and pigmentation of turbot (*Scophthalmus maximus*) larvae fed live-prey enriched in arachidonic and eicosapentaenoic acids. *Aquaculture* 180(3): 321-343.

Evjemo, J. O. & Olsen, Y. (1997). Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. In *Live Food in Aquaculture*, 159-162: Springer.

Evjemo, J. O., Reitan, K. I. & Olsen, Y. (2003). Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value. *Aquaculture* 227(1): 191-210.

Falconer, D. (1960). Introduction to quantitative genetics. London: Oliver and Boyd.

FAO (2015). The State of World Fisheries and Aquaculture. Rome: Food and Agriculture. *Organization of the United Nations*: 1-243.

Faustino, M. & Power, D. (1998). Development of osteological structures in the sea bream: vertebral column and caudal fin complex. *Journal of Fish Biology* 52(1): 11-22.

FEAP (2016). European Aquaculture Production Report. *Federation of European Aquaculture Producers, Liege, Belgium.*

Ferguson, R., Merrifield, D. L., Harper, G. M., Rawling, M. D., Mustafa, S., Picchietti, S., Balcàzar, J. L. & Davies, S. J. (2010). The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *Journal of Applied Microbiology* 109(3): 851-862.

Fernández-Díaz, C., Pascual, E. & Yúfera, M. (1994). Feeding behaviour and prey size selection of gilthead seabream, *Sparus aurata*, larvae fed on inert and live food. *Marine Biology* 118(2): 323-328.

Fernandez-Diaz, C. & Yúfera, M. (1997). Detecting growth in gilthead seabream, *Sparus aurata* L., larvae fed microcapsules. *Aquaculture* 153(1): 93-102.

Fernández-Díaz, C., Yýfera, M., Cañavate, J., Moyano, F., Alarcón, F. & Díaz, M. (2001). Growth and physiological changes during metamorphosis of Senegal sole reared in the laboratory. *Journal of Fish Biology* 58(4): 1086-1097.

Fernández, I. & Gisbert, E. (2011). The effect of vitamin A on flatfish development and skeletogenesis: a review. *Aquaculture* 315(1): 34-48.

Fernández, I., Pimentel, M. S., Ortiz-Delgado, J. B., Hontoria, F., Sarasquete, C., Estévez, A., Zambonino-Infante, J. L. & Gisbert, E. (2009). Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality. *Aquaculture* 295(3): 250-265.

Fisheries - European Commission (2014). Facts and Figures on the Common Fisheries Policy. 1-52.

Folkvord, A. & Hunter, J. (1986). Size-specific vulnerability of northern anchovy, *Engraulis mordax*, larvae to predation by fishes. *Fishery Bulletin* 84(4): 859-869.

Forne, I., Castellana, B., Marín-Juez, R., Cerda, J., Abián, J. & Planas, J. V. (2011). Transcriptional and proteomic profiling of flatfish (*Solea senegalensis*) spermatogenesis. *Proteomics* 11(11): 2195-2211.

Forsythe, J. W. & Van Heukelen, W. F. (1987). Growth. In: Cephalopod life cycles, Comparative Reviews (Boyle, P. R., ed.). *Academic Press, London*: 135–156.

Fulks, W. & Main, K. L. (1991). Rotifer and microalgae culture systems: *proceedings of a US-Asia workshop, Honolulu, Hawaii, January 28-31, 1991. Oceanic Institute*.

Fulton, T.W., 1904. The rate of growth of fishes. 22nd Ann. Rep. Fish. Board Scotland 3:141-241.

García, J. G. & García, B. G. (2006). An econometric viability model for ongrowing sole (*Solea senegalensis*) in tanks using pumped well sea water. *Spanish Journal of Agricultural Research* 4(4): 304-315.

Gavaia, P. J., Dinis, M. T. & Cancela, M. (2002). Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared Senegal sole (*Solea senegalensis*). *Aquaculture* 211(1): 305-323.

Gavaia, P. J., Domingues, S., Engrola, S., Drake, P., Sarasquete, C., Dinis, M. T. & Cancela, M. L. (2009). Comparing skeletal development of wild and hatchery-reared Senegalese sole (*Solea senegalensis*, Kaup 1858): evaluation in larval and postlarval stages. *Aquaculture Research* 40(14): 1585-1593.

Gavaia, P. j., Sarasquete, C. & Cancela, M. L. (2000). Detection of mineralized structures in early stages of development of marine Teleostei using a modified alcian blue-alizarin red double staining technique for bone and cartilage. *Biotechnic & histochemistry* 75(2): 79-84.

Geffen, A., Van der Veer, H. & Nash, R. (2007). The cost of metamorphosis in flatfishes. *Journal of Sea Research* 58(1): 35-45.

Gillen, A. L., Stein, R. A. & Carline, R. F. (1981). Predation by pellet-reared tiger muskellunge on minnows and bluegills in experimental systems. *Transactions of the American Fisheries Society* 110(2): 197-209.

Govoni, J. J., Boehlert, G. W. & Watanabe, Y. (1986). The physiology of digestion in fish larvae. *Environmental Biology of Fishes* 16(1-3): 59-77.

Graeve, M., Kattner, G. & Piepenburg, D. (1997). Lipids in Arctic benthos: does the fatty acid and alcohol composition reflect feeding and trophic interactions? *Polar Biology* 18(1): 53-61.

Guzmán, J. M., Norberg, B., Ramos, J., Mylonas, C. C. & Mañanós, E. L. (2008). Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). *General and Comparative Endocrinology* 156(2): 285-297.

Guzmán, J. M., Ramos, J., Mylonas, C. C. & Mañanós, E. L. (2009). Spawning performance and plasma levels of GnRHa and sex steroids in cultured female

Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. *Aquaculture* 291(3): 200-209.

Gwak, W. & Tanaka, M. (2001). Developmental change in RNA: DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. *Journal of Fish Biology* 59(4): 902-915.

Haga, Y., Du, S.-J., Satoh, S., Kotani, T., Fushimi, H. & Takeuchi, T. (2011). Analysis of the mechanism of skeletal deformity in fish larvae using a vitamin A-induced bone deformity model. *Aquaculture* 315(1): 26-33.

Hagiwara, A. (1996). Appearance of floating resting eggs in the rotifers *Brachionus plicatilis* and *B. rotundiformis*. *Bulletin of the Faculty of Fisheries, Nagasaki University* 77: 111-115.

Hagiwara, A., Kotani, T., Snell, T. W., Assava-Aree, M. & Hirayama, K. (1995). Morphology, reproduction, genetics, and mating behavior of small, tropical marine Brachionus strains (Rotifera). *Journal of Experimental Marine Biology and Ecology* 194(1): 25-37.

Hall, B. K. (2005). *Bones and cartilage: developmental and evolutionary skeletal biology. Academic Press.*

Hall, T. E., Smith, P. & Johnston, I. A. (2004). Stages of embryonic development in the Atlantic cod *Gadus morhua*. *Journal of Morphology* 259(3): 255-270.

Hansen, B., Wernberg-Moller, T. & Wittrup, L. (1997). Particle grazing efficiency and specific growth efficiency of the rotifer *Brachionus plicatilis* (Muller). *Journal of Experimental Marine Biology and Ecology* 215(2): 217-233.

Hart, P. J. & Hamrin, S. F. (1990).The role of behaviour and morphology in the selection of prey by pike. In *Behavioural mechanisms of food selection*, 235-254: Springer.

Haylor, G. (1993). Controlled hatchery production of *Clarias gariepinus* (Burchell, 1822): an estimate of maximum daily feed intake of *C. gariepinus* larvae. *Aquaculture Research* 24(4): 473-482.

Healey, E. (1999). The skin pattern of young plaice and its rapid modification in response to graded changes in background tint and pattern. *Journal of Fish Biology* 55(5): 937-971.

Helland, S., Terjesen, B. F. & Berg, L. (2003). Free amino acid and protein content in the planktonic copepod *Temora longicornis* compared to *Artemia franciscana*. *Aquaculture* 215(1): 213-228.

Helvik, J. V., Drivenes, Ø., Harboe, T. & Seo, H.-C. (2001). Topography of different photoreceptor cell types in the larval retina of Atlantic halibut (*Hippoglossus hippoglossus*). *Journal of Experimental Biology* 204(14): 2553-2559.

Hilton, Z., Poortenaar, C. W. & Sewell, M. A. (2008). Lipid and protein utilisation during early development of yellowtail kingfish (*Seriola lalandi*). *Marine Biology* 154(5): 855-865.

Hino, A. & Hirano, R. (1976). Ecological studies on the mechanism of bisexual reproduction in the rotifer *Brachionus plicatilis*. I. General aspects of bisexual reproduction inducing factors. *日本水産学会誌 = Fisheries society of Japan 42(*10): 1093-1099.

Hino, A. & Hirano, R. (1980). Relationship between body size of the rotifer *Brachionus plicatilis* and the maximum size of particles ingested. *Bulletin of the Japanese Society of Scientific Fisheries* 46(10): 1217-1222.

Hirano, K. (1987). Studies on the culture of the rotifer (*Brachionus plicatilis* OF Mueller). *Bulletin of the Faculty of Agriculture-Miyazaki University (Japan)*.

Hirata, H. (1979). Rotifer culture in Japan. *Special Publication European Mariculture Society* 4: 361-375.

Hirayama, K. & Rumengann, I. F. (1993). The fecundity patterns of S and L type rotifers of *Brachionus plicatilis*. *Hydrobiologia* 255(1): 153-157.

Holland, D. (1978). Lipid reserves and energy metabolism in the larvae of benthic marine invertebrates. *Biochemical and Biophysical Perspectives in Marine Biology* 4: 85-123.

Hosoya, K. & Kawamura, K. (1998). Skeletal formation and abnormalities in the caudal complex of the Japanese Flounder, *Paralichthys olivaceus* (Temminck & Schlegel). *Bulletin-National Research Institute of Fisheries Science*: 97-110.

Hossain, M. A., Tanaka, M. & Masuda, R. (2002). Predator–prey interaction between hatchery-reared Japanese flounder juvenile, *Paralichthys olivaceus*, and sandy

shore crab, *Matutalunaris*: daily rhythms, anti-predator conditioning and starvation. *Journal of Experimental Marine Biology and Ecology* 267(1): 1-14.

Houde, E. (1997). Patterns and trends in larval-stage growth and mortality of teleost fish*. *Journal of Fish Biology* 51(sA): 52-83.

Howell, B. (1972). Preliminary experiments on the rearing of larval lemon sole *Microstomus kitt* (Walbaum) on cultured foods. *Aquaculture* 1: 39-44.

Howell, B. (1973). Marine fish culture in Britain VIII. A marine rotifer, *Brachionus plicatilis* Muller, and the larvae of the mussel, *Mytilus edulis* L., as foods for larval flatfish. *Journal du Conseil* 35(1): 1-6.

Howell, B., Beard, T. & Hallam, J. (1995). The effect of diet quality on the low-temperature tolerance of juvenile sole, *Solea solea* (L.). *ICES CM*.

Howell, B., Cañavate, J., Prickett, R. & Conceicao, L. (2006). Farming soles a reality at last. *Aquaculture European World Aquaculture* 37: 3-6.

Howell, B., Pricket, R., Cañavate, P., Mañanos, E., Dinis, M., Conceição, L. & Valente, L. (2011a). Report of the 5th Workshop on the Cultivation of Soles. *Centre of Marine Sciences (CCMAR), University of the Algarve, Faro, Portugal*: 12.

Howell, B., Prickett, R., Cañavate, J. P., Mañanós, E. L., Dinis, M., Conceição, L. & Valente, L. (2011b). Sole farming: There or thereabouts! *Aquaculture* 36: 42-45.

Howell, B. R. (1997). A re-appraisal of the potential of the sole, *Solea solea* (L.), for commercial cultivation. *Aquaculture* 155(1): 355-365.

Howell, B. R., Conceição, L. E. C., Prickett, R., Cañavate, J. P., & Mañanos, E. L (2009). *Report of the 4th Workshop on the Cultivation of Soles . In Workshop on the Cultivation of Soles.* CCMAR, University of Algarve, Faro, Portugal, pp.47.

Hoyle, J. A. & Keast, A. (1987). The effect of prey morphology and size on handling time in a piscivore, the largemouth bass (*Micropterus salmoides*). *Canadian Journal of Zoology* 65(8): 1972-1977.

Ikenoue, H. & Kafuku, T. (1992). Modern methods of aquaculture in Japan. *Development in Aquaculture and Fisheries Science*, 11. Kodansha, Tokyo; Elsevier, Amsterdam.

Imsland, A., Foss, A., Conceiçao, L. E., Dinis, M. T., Delbare, D., Schram, E., Kamstra, A., Rema, P. & White, P. (2003). A review of the culture potential of *Solea solea* and *S. senegalensis*. *Reviews in Fish Biology and Fisheries* 13(4): 379-408.

Izquierdo, M., Arakawa, T., Takeuchi, T., Haroun, R. & Watanabe, T. (1992). Effect of n-3 HUFA levels in Artemia on growth of larval Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 105(1): 73-82.

Izquierdo, M., Socorro, J., Arantzamendi, L. & Hernández-Cruz, C. (2000). Recent advances in lipid nutrition in fish larvae. *Fish Physiology and Biochemistry* 22(2): 97-107.

Johnston, I. & Hall, T. E. (2004).Mechanisms of Muscle Development and Responses to Temperature Change in Fish Larvae. In *American Fisheries Society Symposium*, Vol. 40, 85-116.

Juanes, F., Stouder, D. & Feller, K. (1994). What determines prey size selectivity in piscivorous fishes. In: *Theory and Application in Fish Feeding Ecology*. Carolina University Press, Columbia, p79-100.

Kainz, M., Arts, M. T. & Mazumder, A. (2004). Essential fatty acids in the planktonic food web and their ecological role for higher trophic levels. *Limnology and Oceanography* 49(5): 1784-1793.

Kates, M. & Volcani, B. (1966). Lipid components of diatoms. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* 116(2): 264-278.

Kawamura, G., Masuma, S., Tezuka, N., Koiso, M., Jinbo, T. & Namba, K. (2003). Morphogenesis of sense organs in the bluefin tuna *Thunnus orientalis*. *The Big Fish Bang*: 123-135.

Keast, A. & Webb, D. (1966). Mouth and body form relative to feeding ecology in the fish fauna of a small lake, Lake Opinicon, Ontario. *Journal of the Fisheries Board of Canada* 23(12): 1845-1874.

Kohno, H., Taki, Y., Ogasawara, Y., Shirojo, Y., Taketomi, M. & Inoue, M. (1983). Development of swimming and feeding functions in larval *Pagrus major*. *Gyoruigaku zasshi = Japanese Journal of Ichthyology 30, 47-60*.

Koumoundouros, G. (2010). Morpho-anatomical abnormalities in Mediterranean marine aquaculture. *Recent Advances in Aquaculture Research*: 125-148.

Koumoundouros, G., Divanach, P. & Kentouri, M. (1999). Osteological development of the vertebral column and of the caudal complex in *Dentex dentex*. *Journal of Fish Biology* 54(2): 424-436.

Koumoundouros, G., Gagliardi, F., Divanach, P., Boglione, C., Cataudella, S. & Kentouri, M. (1997). Normal and abnormal osteological development of caudal fin in *Sparus aurata* L. fry. *Aquaculture* 149(3): 215-226.

Kvenseth, A., Pittman, K. & Helvik, J. (1996). Eye development in Atlantic halibut (*Hippoglossus hippoglossus*): differentiation and development of the retina from early yolk sac stages through metamorphosis. *Canadian Journal of Fisheries and Aquatic Sciences* 53(11): 2524-2532.

Lagardère, F. (1979). Ichtyoplancton de *Solea senegalensis* Kaup, 1858 (Soleidae-Pleuronectiformes) Description des ouefs-Problèmes posès par l'identification des larves. *Annales de la Société des Sciences Naturelles de la Charente-Maritime* 6: 583-596.

Le Milinaire, C. (1984).Étude du besoin en acides gras essentiels pour la larve de turbot (*Psetta maxima* L.) pendant la phase d'alimentation avec le rotifère *Brachionus plicatilis* (OF Muller). *These 3eme CycleUniversité Bretagne Occidentale*, 167pp.

Lebegue, E. (1982). Etude morphologique et experimentale sur la pigmentation de larves et de juveniles de sole (*Solea Vulgaris* Q.) et de turbot (*Psetta maxima* L.). *PhD thesis, Universite de Bretagne Occidentale, France.*

Léger, P., Bengtson, D., Simpson, K. & Sorgeloos, P. (1986). The use and nutritional value of Artemia as a food source. *Oceanogr. Mar. Biol. Ann. Rev* 24: 521-623.

Lie, O., Haaland, H., Hemre, G.-I., Maage, A., Lied, E., Rosenlund, G., Sandnes, K. & Olsen, Y. (1997). Nutritional composition of rotifers following a change in diet from yeast and emulsified oil to microalgae. *Aquaculture International* 5(5): 427-438.

Lubzens, E., Minkoff, G. & Marom, S. (1985). Salinity dependence of sexual and asexual reproduction in the rotifer *Brachionus plicatilis*. *Marine Biology* 85(2): 123-126.

Lubzens, E., Rothbard, S., Blumenthal, A., Kolodny, G., Perry, B., Olund, B., Wax, Y. & Farbstein, H. (1987). Possible use of *Brachionus plicatilis* (OF Müller) as food for freshwater cyprinid larvae. *Aquaculture* 60(2): 143-155.

Lubzens, E., Tandler, A. & Minkoff, G. (1989). Rotifers as food in aquaculture. *Hydrobiologia* 186(1): 387-400.

Lubzens, E., Wax, Y., Minkoff, G. & Adler, F. (1993). A model evaluating the contribution of environmental factors to the production of resting eggs in the rotifer *Brachionus plicatilis. Hydrobiologia* 255(1): 127-138.

Lubzens, E., Zmora, O. & Barr, Y. (2001).Biotechnology and aquaculture of rotifers. In *Rotifera IX*, 337-353: Springer.

Lund, I., Steenfeldt, S. J., Banta, G. & Hansen, B. W. (2008). The influence of dietary concentrations of arachidonic acid and eicosapentaenoic acid at various stages of larval ontogeny on eye migration, pigmentation and prostaglandin content of common sole larvae (*Solea solea* L.). *Aquaculture* 276(1): 143-153.

Maeda, M., Nogami, K., Kanematsu, M. & Hirayama, K. (1997). The concept of biological control methods in aquaculture. *Hydrobiologia* 358(1-3): 285-290.

Magalhães, N. & Dinis, M. (1996).The effect of starvation and feeding regimes on the RNA, DNA and protein content of *Solea senegalensis* larvae. In *Book of Abstracts World Aquaculture*, Vol. 5, 242-248.

Mañanos, E. (2011).Hormonal control of reproduction in Senegalese sole. In *5 th Workshop on the Cultivation of Soles CCMAR, University of the Algarve, Faro, Portugal*, 5-7.

Mañanós, E., Duncan, N. & Mylonas, C. (2008). Reproduction and control of ovulation, spermiation and spawning in cultured fish. *Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC Press, Taylor and Francis Group, Boca Raton*: 3-80.

Marin-Magan, V., Anguis, V. & Canavete, J. (1995).Uso de alimento inerte en larvas y alevines del lenguado *Solea senegalensis*. In *Actas del V Congreso Nacional de Acuicultura*, 10-13.

Marino, G., Boglione, C., Bertolini, B., Rossi, A., Ferreri, F. & Cataudella, S. (1993). Observations on development and anomalies in the appendicular skeleton of sea bass, *Dicentrarchus labrax* L. 1758, larvae and juveniles. *Aquaculture Research* 24(3): 445-456.

Martinez, I., Moyano, F., Fernández-Díaz, C. & Yúfera, M. (1999). Digestive enzyme activity during larval development of the Senegal sole (*Solea senegalensis*). *Fish Physiology and Biochemistry* 21(4): 317-323.

McGurk, M. (1984). Effects of delayed feeding and temperature on the age of irreversible starvation and on the rates of growth and mortality of Pacific herring larvae. *Marine Biology* 84(1): 13-26.

Miller, T. J., Crowder, L. B., Rice, J. A. & Marschall, E. A. (1988). Larval size and recruitment mechanisms in fishes: toward a conceptual framework. *Canadian Journal of Fisheries and Aquatic Sciences* 45(9): 1657-1670.

Morais, S., Narciso, L., Dores, E. & Pousao-Ferreira, P. (2004). Lipid enrichment for Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and fatty acid profile. *Aquaculture International* 12(3): 281-298.

Morehead, D. T., Battaglene, S. C., Metillo, E. B., Bransden, M. P. & Dunstan, G. A. (2005). Copepods as a live feed for striped trumpeter *Latris lineata* larvae. *Copepods in Aquaculture*: 195-208.

Mourente, G. (2003). Accumulation of DHA (docosahexaenoic acid; 22: 6n-3) in larval and juvenile fish brain. *The big fish bang. Institute of Marine Research, Bergen*: 239-248.

Munk, P. (1997). Prey size spectra and prey availability of larval and small juvenile cod. *Journal of Fish Biology* 51(sA): 340-351.

Næss, T. & Lie, Ø. (1998). A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. *Aquaculture Research* 29(12): 925-934.

Navarro, J. C., Henderson, R. J., McEvoy, L. A., Bell, M. V. & Amat, F. (1999). Lipid conversions during enrichment of Artemia. *Aquaculture* 174(1): 155-166.

Nielsen, L. A. (1980). Effect of walleye (*Stizostedion vitreum*) predation on juvenile mortality and recruitment of yellow perch (*Perca flavescens*) in Oneida Lake, New York. *Canadian Journal of Fisheries and Aquatic Sciences* 37(1): 11-19.

Noble, C., Jones, H. A. C., Damsgård, B., Flood, M. J., Midling, K. Ø., Roque, A., Sæther, B.-S. & Cottee, S. Y. (2012). Injuries and deformities in fish: their potential impacts

upon aquacultural production and welfare. *Fish Physiology and Biochemistry* 38(1): 61-83.

Nogrady, T., Wallace, R. & Snell, T. (1993). Guides to the Identification of the Microinvertebrates of the Continental Waters of the World. *The Hague: SPB Academic Publishing* 1.

Norambuena, F. (2012).Senegalese sole (*Solea senegalensis*) broodstock nutrition: arachidonic acid (20: 4n-6, ARA) and reproductive physiology. *PhD Thesis, Autonomous University of Barcelona, Barcelona Spain.*

Olsen, A. I., Attramadal, Y., Reitan, K. I. & Olsen, Y. (2000). Food selection and digestion characteristics of Atlantic halibut (*Hippoglossus hippoglossus*) larvae fed cultivated prey organisms. *Aquaculture* 181(3): 293-310.

Østergaard, P., Munk, P. & Janekarn, V. (2005). Contrasting feeding patterns among species of fish larvae from the tropical Andaman Sea. *Marine Biology* 146(3): 595-606.

Padrós, F., Villalta, M., Gisbert, E. & Estévez, A. (2011). Morphological and histological study of larval development of the Senegal sole *Solea senegalensis*: an integrative study. *Journal of Fish Biology* 79(1): 3-32.

Palazzi, R., Richard, J., Bozzato, G. & Zanella, L. (2006). Larval and juvenile rearing of common sole (*Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture* 255(1): 495-506.

Pankhurst, P. & Eagar, R. (1996). Changes in visual morphology through life history stages of the New Zealand snapper, *Pagrus auratus*. *New Zealand Journal of Marine and Freshwater Research* 30(1): 79-90.

Parra, G. (1998).Fisiología y Balance Energético Durante el Desarrollo Larvario de Peces Marinos *(Solea senegalensis* Kaup and *Sparus autara* Linneo) en Cultivo. *Tesis doctoral, Universidad de Cádiz.*

Parra, G., Rønnestad, I. & Yúfera, M. (1999). Energy metabolism in eggs and larvae of the Senegal sole. *Journal of Fish Biology* 55(sA): 205-214.

Parra, G. & Yúfera, M. (2001). Comparative energetics during early development of two marine fish species, *Solea senegalensis* (Kaup) and *Sparus aurata* (L.). *Journal of Experimental Biology* 204(12): 2175-2183.

Persson, L. (1990).Predicting ontogenetic niche shifts in the field: what can be gained by foraging theory? In *Behavioural mechanisms of food selection*, 303-321: Springer.

Polo, A., Yúfera, M. & Pascual, E. (1992). Feeding and growth of gilthead seabream (*Sparus aurata* L.) larvae in relation to the size of the rotifer strain used as food. *Aquaculture* 103(1): 45-54.

Pontin, R. M. (1978). A key to the freshwater planktonic and semi-planktonic Rotifera of the British Isles. *Freshwater Biological Association Ambleside, Scientific Publication* 38, Cumbria.

Popova, O. (1967). The 'predator-prey' relationship among fish. In: *Gerking SD (ed) The biological basis of freshwater fish production. Blackwell Scientific Publications, Oxford,* p 359–376.

Popova, O. (1978). The role of predaceous fish in ecosystems. In: Gerking SD (ed) Ecology of freshwater fish production. John Wiley & Sons, New York, p 215–249.

Porta, J., Porta, J. M., Martínez-Rodríguez, G. & Alvarez, M. C. (2006). Genetic structure and genetic relatedness of a hatchery stock of Senegal sole (*Solea senegalensis*) inferred by microsatellites. *Aquaculture* 251(1): 46-55.

Pourriot, R. (1977). Food and feeding habits of Rotifera. *Arch. Hydrobiol. Beih* 8: 243-260.

Quéro, J.-C. & Vayne, J.-J. (1997). Les poissons de mer des pêches françaises:[identification, inventaire et répartition de 209 espèces]. *Delachaux et Niestlé.*

Reig, L., Oca, J. & Flos, R. (2000). Trading perspectives of a potential farmed sole (*Solea sp.*) evaluated at the Central Fish Market of Barcelona (Spain). *Responsible Aquaculture in the New Millennium. EAS Special Publication* 28: 596.

Ribeiro, L., Sarasquete, C. & Dinis, M. (1999a). Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larvae. *Aquaculture* 171(3): 293-308.

Ribeiro, L., Zambonino-Infante, J., Cahu, C. & Dinis, M. (1999b). Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture* 179(1): 465-473.

Ribeiro, L., Zambonino-Infante, J., Cahu, C. & Dinis, M. (2002). Digestive enzymes profile of *Solea senegalensis* post larvae fed Artemia and a compound diet. *Fish Physiology and Biochemistry* 27(1-2): 61-69.

Rijnsdorp, A., Van Beek, F., Flatman, S., Millner, R., Riley, J., Giret, M. & De Clerck, R. (1992). Recruitment of sole stocks, *Solea solea* (L.), in the Northeast Atlantic. *Netherlands Journal of Sea Research* 29(1): 173-192.

Rodríguez Martínez, R. B. (1984). Biología y cultivo de *Solea Senegalensis* Kaup, 1858 en el Golfo de Cádiz. *PhD Thesis, Facultad de Biología, Universidad de Sevilla.*

Rombaut, G., Dhert, P., Vandenberghe, J., Verschuere, L., Sorgeloos, P. & Verstraete, W. (1999). Selection of bacteria enhancing the growth rate of axenically hatched rotifers (*Brachionus plicatilis*). *Aquaculture* 176(3): 195-207.

Rosenlund, G., Stoss, J. & Talbot, C. (1997). Co-feeding marine fish larvae with inert and live diets. *Aquaculture* 155(1): 183-191.

Rothhaupt, K. (1990a). Differences in particle size-dependent feeding efficiencies of closely related rotifer species. *Limnology and Oceanography* 35(1): 16-23.

Rothhaupt, K. O. (1990b). Changes of the functional responses of the rotifers *Brachionus rubens* and *Brachionus calyciflorus* with particle sizes. *Limnology and Oceanography* 35(1): 24-32.

Rothhaupt, K. O. (1990c). Population growth rates of two closely related rotifer species: effects of food quantity, particle size, and nutritional quality. *Freshwater Biology* 23(3): 561-570.

Ruane, N., Makridis, P., Balm, P. & Dinis, M. (2005). Skin darkness is related to cortisol, but not MSH, content in post-larval *Solea senegalensis*. *Journal of Fish Biology* 67(2): 577-581.

Rueda-Jasso, R., Conceiçao, L. E., Dias, J., De Coen, W., Gomes, E., Rees, J.-F., Soares, F., Dinis, M. T. & Sorgeloos, P. (2004). Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture* 231(1): 417-433.

Rumengan, I. & Hirayama, K. (1990).Growth responses of genetically distinct S and L type rotifer (*Brachionus plicatilis*) strains to different temperatures. In *The second Asian fisheries forum*, 33-36: *Asian Fisheries Society.*

Rust, M. B. (1995).Quantitative aspects of nutrient assimilation in six species of fish larvae. *University of Washington Seattle, USA.*

Ruttner-Kolisko, A. (1974). Plankton rotifers. Biology and Taxonomy (Die Binnengewasser vol. XXVI; Part 1: Chapter 'Die Rotatorien') Stuttgart. *English translation*: 99-234.

Salas-Leiton, E., Anguis, V., Manchado, M. & Canavate, J. P. (2008). Growth, feeding and oxygen consumption of Senegalese sole (*Solea senegalensis*) juveniles stocked at different densities. *Aquaculture* 285(1): 84-89.

Sargent, J., Bell, J., Bell, M., Henderson, R. & Tocher, D. (1995). Requirement criteria for essential fatty acids. *Journal of Applied Ichthyology* 11(3-4): 183-198.

Sargent, J. & Falk-Petersen, S. (1988). The lipid biochemistry of calanoid copepods. In *Biology of Copepods*, 101-114: Springer.

Sargent, J., McEvoy, L., Estevez, A., Bell, G., Bell, M., Henderson, J. & Tocher, D. (1999). Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture* 179(1): 217-229.

Sargent, J. R., Tocher, D. R. & Bell, J. G. (2002). The lipids. *Fish nutrition* 3: 181-257.

Serra, M., Gómez, Á. & Carmona, M. J. (1998). Ecological genetics of Brachionus sympatric sibling species. *Hydrobiologia* 387: 373-384.

Serra, M. & King, C. (1999). Optimal rates of bisexual reproduction in cyclical parthenogens with density-dependent growth. *Journal of Evolutionary Biology* 12(2): 263-271.

Shand, J., Hart, N. S., Thomas, N. & Partridge, J. C. (2002). Developmental changes in the cone visual pigments of black bream *Acanthopagrus butcheri*. *Journal of Experimental Biology* 205(23): 3661-3667.

Shirota, A. (1970). Studies on the mouth size of fish larvae. *Bulletin of the Japanese Society of Scientific Fisheries* 36, 353–368.

Skjermo, J. & Vadstein, O. (1999). Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture* 177(1): 333-343.

Snell, T. (1986). Effect of temperature, salinity and food level on sexual and asexual reproduction in *Brachionus plicatilis* (Rotifera). *Marine Biology* 92(2): 157-162.

Snell, T. W. & Boyer, E. M. (1988). Thresholds for mictic female production in the rotifer *Brachionus plicatilis* (Muller). *Journal of Experimental Marine Biology and Ecology* 124(2): 73-85.

Solbakken, J., Norberg, B., Watanabe, K. & Pittman, K. (1999). Thyroxine as a Mediator of Metamorphosis of Atlantic Halibut, *Hippoglossus Hippoglossus*. *Environmental Biology of Fishes* 56(1-2): 53-65.

Srivastava, A., Hamre, K., Stoss, J., Chakrabarti, R. & Tonheim, S. K. (2006). Protein content and amino acid composition of the live feed rotifer (*Brachionus plicatilis*): with emphasis on the water soluble fraction. *Aquaculture* 254(1): 534-543.

Støttrup, J. (2000). The elusive copepods: their production and suitability in marine aquaculture. *Aquaculture Research* 31(8-9): 703-711.

Taki, Y., Kohno, H. & Hara, S. (1987). Morphological aspects of the development of swimming and feeding functions in the milkfish *Chanos chanos*. *Japanese Journal of Ichthyology* 34(2): 198-208.

Tzoumas, A. (1988).The Nutritional Value of Freshly Hatched and Enriched Artemia Nauplii for Sole Larvae (*Solea Solea)*, from the Standpoint of Their Poly-unsaturated Fatty Acid Content. University of Stirling.

Vázquez, R., González, S., Rodriguez, A. & Mourente, G. (1994). Biochemical composition and fatty acid content of fertilized eggs, yolk sac stage larvae and first-feeding larvae of the Senegal sole (*Solea senegalensis* Kaup). *Aquaculture* 119(2): 273-286.

Verreth, J. (1994). Nutrition and related ontogenetic aspects in larvae of the african catfish *Clarias gariepinus*. *DsC thesis, Department of Fish Culture and Fisheries, Wageningen Agricultural University, Wageningen, The Netherlands*.

Villalta, M., Estévez, A. & Bransden, M. P. (2005a). Arachidonic acid enriched live prey induces albinism in Senegal sole (*Solea senegalensis*) larvae. *Aquaculture* 245(1): 193-209.

Villalta, M., Estévez, A., Bransden, M. P. & Bell, J. G. (2005b). The effect of graded concentrations of dietary DHA on growth, survival and tissue fatty acid profile of Senegal sole (*Solea senegalensis*) larvae during the Artemia feeding period. *Aquaculture* 249(1): 353-365.

Vinagre, C., Fonseca, V., Cabral, H. & Costa, M. J. (2006). Habitat suitability index models for the juvenile soles, *Solea solea* and *Solea senegalensis*, in the Tagus estuary: Defining variables for species management. *Fisheries Research* 82(1): 140-149.

Wahl, C. M., Mills, E. L., McFarland, W. N. & DeGisi, J. S. (1993). Ontogenetic changes in prey selection and visual acuity of the yellow perch, *Perca flavescens*. *Canadian Journal of Fisheries and Aquatic Sciences* 50(4): 743-749.

Walker, M. G. & Emerson, L. (1990). The seasonal migration of soles (*Solea solea*) through the Dover Strait. *Netherlands Journal of Sea Research* 25(3): 417-422.

Wallace, R., Snell, T., Thorp, J. & Covich, A. (1991). Ecology and classification of North American freshwater invertebrates. *Rotifera*: 187-248.

Webb, P. (1976). The effect of size on the fast-start performance of rainbow trout *Salmo gairdneri*, and a consideration of piscivorous predator-prey interactions. *The Journal of Experimental Biology* 65(1): 157-177.

Whitehead, P. J. P., Bauchot, M.L., Hureau, J.C., Nielsen, J., Tortonese, E. (1986). Fisches of the North-eastern Atlantic and the Mediterrnean. *Unesco, Paris, 1473 pp*.

Yin, M. & Blaxter, J. (1987). Feeding ability and survival during starvation of marine fish larvae reared in the laboratory. *Journal of Experimental Marine Biology and Ecology* 105(1): 73-83.

Yúfera, M. & Darias, M. (2007). The onset of exogenous feeding in marine fish larvae. *Aquaculture* 268(1): 53-63.

Yúfera, M., Fernández-Díaz, C. & Pascual, E. (2005). Food microparticles for larval fish prepared by internal gelation. *Aquaculture* 248(1): 253-262.

Yúfera, M., Parra, G., Santiago, R. & Carrascosa, M. (1999). Growth, carbon, nitrogen and caloric content of *Solea senegalensis* (Pisces: Soleidae) from egg fertilization to metamorphosis. *Marine Biology* 134(1): 43-49.

Internship at the sole hatchery Safiestela, S.A. – Substitution of Instar I with enriched Instar II artemia in the first days of *Solea senegalensis* rearing

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